HUMAN EPIDERMAL DENDRITIC CELLS

An ultrastructural study of human epidermal dendritic cells under physical stress and after malignant change

by

John Angus Alexander Hunter

B.A.(Cantab.) M.B. Ch.B.(Edin.) M.R.C.P.(Edin.)

M.D., University of Edinburgh

1976.

INDEX*

	ABSTRACT	Page 2.
I.	Introduction.	Page 5.
II.	Materials and methods.	Page 62.
III.	Normal skin.	Page 68.
IV.	Ultraviolet irradiation.	Page 88.
V.	Suction injury.	Page 116.
VI.	Friction injury.	Page 132.
VII.	Histiocytosis X.	Page 145.
VIII.	Malignant melanoma.	Page 165.
IX.	"Pseudo-melanoma".	Page 223.
X.	General discussion and conclusions.	Page 237.
XI.	References.	Page 250.
XII.	Acknowledgements.	Page 274.

* Each Chapter is prefaced with an index of that Chapter.

Abstract of Thesis

This thesis describes ultrastructural observations on normal dendritic cells (melanocytes and Langerhans cells) of human epidermis, and changes in their fine structure after various forms of physical stress and malignant transformation.

The historical background and previous research on the structure and function of the melanocyte and Langerhans cell are reviewed. During a holiday in Madeira the grave of Paul Langerhans was found neglected and overgrown, but arrangements made with a chance acquaintance should ensure that it is looked after in perpetuo.

The unique fragility of melanocyte mitochondria was the only new finding noted in studies of normal skin.

Ultrastructural dopa and tyrosine studies were made on skin exposed to ultraviolet radiation. They highlight the central role of the Golgi apparatus and surrounding system of smooth endoplasmic reticulum (GERL) in the transport of tyrosinase from ribosome to melanosome, and indicate that Stage I melanosomes arise from all parts of the Golgi apparatus as well as from GERL. The concept that the fine structure with periodicity in early melanosomes is the supporting structure of tyrosinase was not confirmed. After a single exposure to ultraviolet radiation (6 X minimal

erythema dose), reaction product was not noted in melanocytes until twenty four hours after irradiation. It was then seen within the Golgi apparatus and neighbouring GERL; seventy two hours after irradiation it was seen mainly in peripheral cytoplasmic vesicles. Langerhans and mast cells were consistently dopa negative.

Suction and friction stress to the epidermis have a profound effect on keratinocytes but cause minimal damage to dendritic cells, probably due to their lack of attachment with surrounding cells. Suction experiments indicate that, in contrast to keratinocytes, there is no communication between the extracellular space and system of smooth endoplasmic reticulum in dendritic cells.

Subcellular observations on a single case of histiocytosis X were similar to those of others, and support the view that this condition is due to reactive proliferation of the epidermal Langerhans cell.

Ultrastructural studies were carried out on forty specimens of invasive malignant melanoma. The findings support the concept that the fine structure of lentigo maligna melanoma is usually characteristic, and differs from that of superficial spreading and nodular malignant melanoma. The melanosomes in lentigo maligna melanoma are usually ellipsoidal and resemble those of normal melanocytes, whereas the melanosomes in superficial spreading and nodular melanoma are most often spheroidal and abnormal in appearance. Superficial

spreading and nodular melanomas cannot be reliably distinguished by their ultrastructure, though vacuolar melanosomes are more common in superficial spreading melanoma. There was no support for the hypothesis that superficial spreading melanomas and nodular melanomas develop from pre-existing naevi.

Ultrastructural dopa reactions carried out on ten malignant melanoma specimens indicated that malignant transformation affects the structural protein of the melanosome rather than the distribution and passage of tyrosinase. Vacuolar melanosomes seem to represent an arrest in the development of Stage I melanosomes.

A single case of a breast carcinoma invading the overlying epidermis and causing gross proliferation and activity of the melanocytes is described. Hyperactive melanocytes, which had migrated from the epidermis, were seen surrounding dermal tumour cells, but little evidence of phagocytosis, either by the melanocytes or tumour cells, was noted. This curiosity illustrates the close relationship which exists between melanocytes and cells of epithelial origin.

Chapter I

INTRODUCTION

Including

1. TERMINOLOGY OF DENDRITIC CELLS

2. THE MELANOCYTE
   - a. Cellular site of melanin synthesis.
   - b. Origin of melanocyte.
   - c. Epidermal melanin unit.
   - d. Metabolic pathway in melanin formation.
   - e. Subcellular sites of melanin biosynthesis.
   - f. Ultrastructure of the melanocyte.
   - g. Racial differences in pigmentation.

3. THE LANGERHANS CELL
   - a. Paul Langerhans.
   - b. Histology and histochemistry of the Langerhans cell.
   - c. Ultrastructure of the Langerhans cell.
   - d. Origin of the Langerhans cell.
   - e. Distribution of Langerhans cells in the body.
   - f. Histiocytosis X.
   - g. Function of the Langerhans cell.

4. THE INDETERMINATE DENDRITIC CELL

5. INTRODUCTION TO THE PRESENT WORK

1. TERMINOLOGY OF DENDRITIC CELLS

Light microscopic examination of vertical sections of human skin reveals that the lower and middle epidermis is composed of two types of cell called keratinocytes and clear cells (Plate 1). Keratinocytes (prickle cells, squamous cells), involved in the process of keratinisation, make up the bulk of the epidermis and are joined together by desmosomes (intercellular bridges). They are produced by mitotic activity in the basal cell layers and move towards the skin surface forming the squamous cell layer (stratum Malpighii) and granular cell layer (stratum granulosum). Finally they transform into horny cells which are shed as they reach the surface. It has been estimated that it takes between 26 and 42 days for the keratinocyte to travel from the basal cell layer to the surface of the granular layer (Halprin, 1972) and a further 14 days for the passage of horny cells through the normal horny layer (Frost et al., 1966).

Clear cells are much more sparse. They are distinguished from keratinocytes by the abundance of their poorly stained cytoplasm and lack of desmosomal attachments with surrounding cells. They occur both in the basal cell layer and throughout the squamous cell layer (Plate 1). Unless fixation and processing of the skin is good the cytoplasm is apt to collapse around the nucleus leaving an empty space.

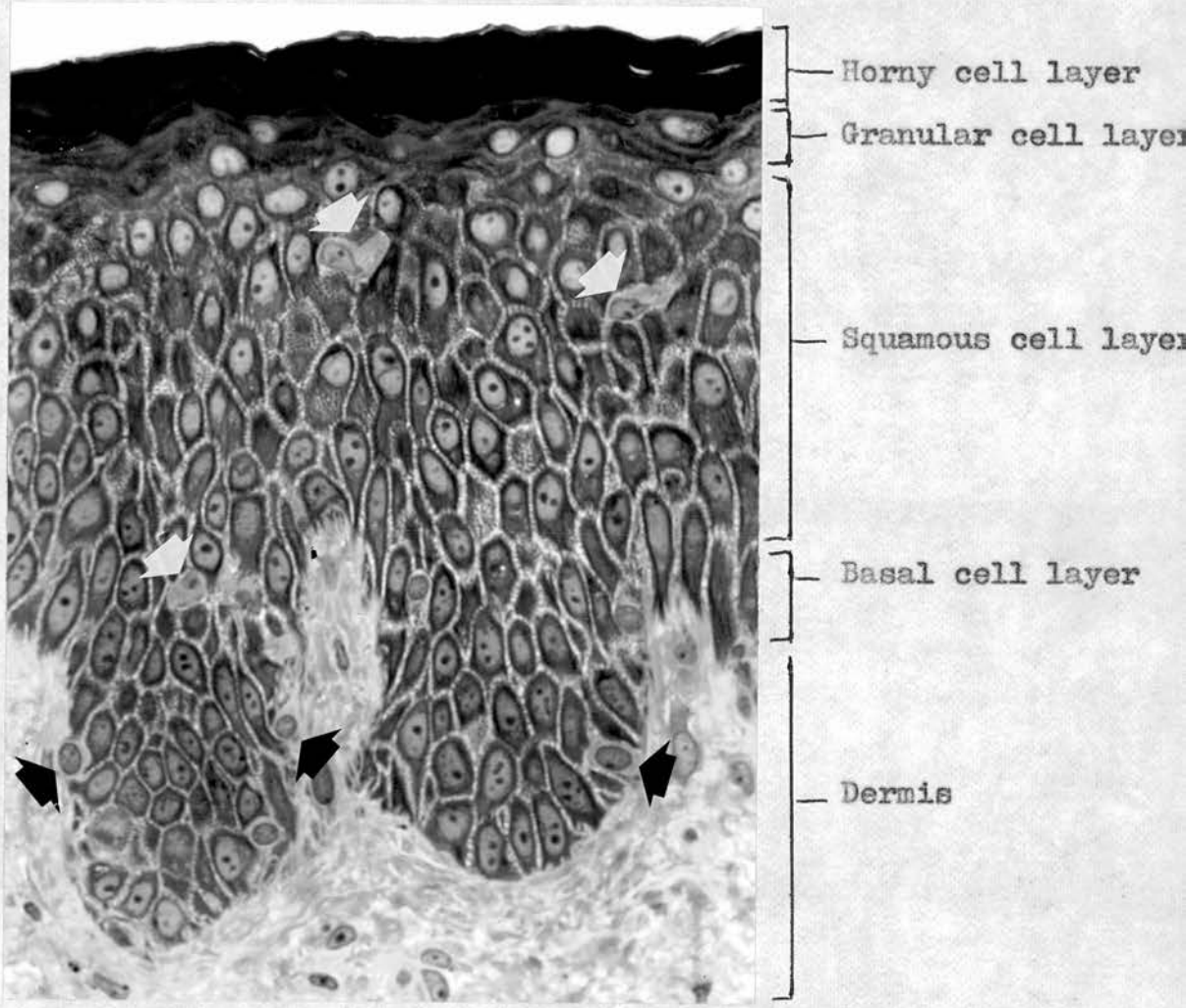


Plate 1 (X540): Vertical section of human epidermis and superficial dermis (lower back).

White arrows point to high level clear cells (probably Langerhans cells) and the black to basal layer clear cells (probably melanocytes).

(Semi thin toluidine blue stained epoxy resin section)

These differences between cells in the epidermis were appreciated as long as 100 years ago. It was also realised that clear cells had branched processes, and that they could be separated into two types depending on whether, or not, they contained pigment. Langerhans (1868) recognised non pigmented gold staining branched cells high in the human epidermis, and Riehl (1884) described migratory pigmented branched cells ("pigmentirten wanderzellen") in human hair papillae.

The synonymy of the branched cells of the epidermis is of bewildering complexity. "Dendritic cell" (Becker, 1927) is a purely descriptive term and is useful for describing all cells of the epidermis with numerous branched processes. Three types are now recognised, and they are most commonly called:

1. melanocyte (the pigmented dendritic cell described by Riehl)
2. Langerhans cell (named eponymously after the original description of Langerhans)
3. indeterminate cell (appreciated more recently, as it shows neither the ultrastructural characteristics of melanocytes nor Langerhans cells. See page 59.)

The word 'melanocyte' became accepted on a world wide basis only after Fitzpatrick and Lerner (1953) pointed out that investigators in biology and medicine were using different terms for the same cells. They recommended the following terminology for pigment cells:

Mature melanin-forming cell - Melanocyte

Immature melanin-forming cell - Melanoblast

Cell with phagocytized melanin - Macrophage
(or Melanophage)

(Melas, Gr. black; kytos, Gr. hollow vessel
blastos, Gr. germ; phagein, Gr. to eat)

2. THE MELANOCYTE

a. Cellular site of melanin synthesis

Bloch's discovery of the reaction between 3,4-dioxyphenylalanin, called dopa for short, and an oxidizing factor in pigment-forming cells was a most significant step forward (Bloch, 1917). He noted that pigmented dendritic cells exposed to dopa showed diffuse blackening of their entire cytoplasm and attributed this to a ferment which he called 'dopa-oxydase'. Bloch considered that non dendritic cells (keratinocytes) could transform into dendritic cells if given the necessary functional stimulation (i.e. need for excessive pigment production) though others felt that dendritic and non dendritic cells were genetically different.

Improvements in Bloch's dopa technique (Becker et al., 1935) led the Canadian Masson (1948) to state his belief that the dopa positive melanocyte was the only melanin-producing cell in the epidermis, and his pioneer work was confirmed and amplified by Billingham (1948).

Billingham (1948) showed that the branches of melanocytes, seen in the basal layer, travelled along the intercellular spaces between ordinary epidermal cells, split frequently, and ultimately terminated in the form of 'caps' or 'end-buttons' closely applied to the walls of ordinary epidermal cells. He felt that

pigment granules were elaborated within the melanocyte and passed to the surrounding epidermal cells across these end caps. Fifteen years later Fitzpatrick and Breathnach (1963) were to suggest the term "Epidermal Melanin Unit" for the melanocyte and its surrounding keratinocytes (see page 12). In 1949 Billingham pointed out that the pigmented epidermis of Indian and Negro skin was similar anatomically to that of white skin. The increased pigmentation was due not to an increased number of melanocytes but rather to a higher rate of melanin production in individual cells. Some years later Cruikshank and Harcourt (1964) were able to confirm the process of pigment donation <u>in vitro</u> by melanocytes in elegant cinematographic studies.

b. <u>Origin of the melanocyte</u>

Experimental work on mice led the embryologist Rawles to conclude that the pigment-forming cells arise from the neural crest and not from neighbouring epidermal cells (Rawles, 1948). By 1950, therefore, it was appreciated that there was only one type of cell in the epidermis capable of manufacturing pigment and that this cell had a cell-lineage of its own and was not derived from keratinocytes of the basal layer.

c. <u>Epidermal Melanin Unit</u>

This term was originally introduced by Fitzpatrick

and Breathnach (1963) to describe a structural, as well as functional, unit of the melanocyte and surrounding keratinocytes (Fig. 1). Its use should emphasise the fact that pigmentary processes involve not only the melanocyte but also the surrounding keratinocytes which acquire the pigment secondarily.

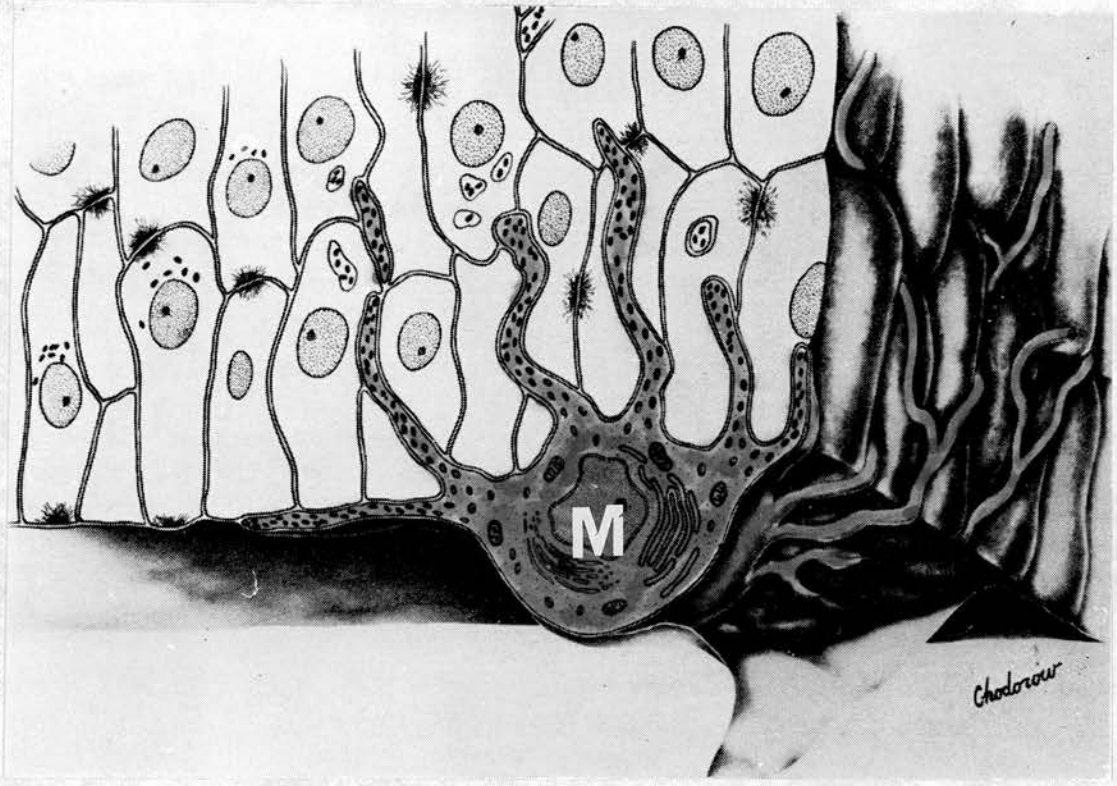


Figure 1: The epidermal melanin unit.
Dendritic processes of a basal melanocyte (M) are seen winding between surrounding keratinocytes.
(From Fitzpatrick et al., 1967)

d. Metabolic pathway in melanin formation

Knowledge of the biochemistry of melanin formation in plants and invertebrates preceeded any real understanding of the problem in man. In 1895 Bourqueolot and Bertrand discovered tyrosinase in a black toadstool Russula Nigricans. Soon afterwards it was appreciated that the enzyme was widely distributed in the vegetable kingdom. In the early 1920's, a few years after Bloch's discovery of dopa-oxidase by histochemical means, Raper and his colleagues in Manchester carried out extensive studies on the metabolic pathway involved in the conversion of tyrosine to melanin in plants. The pathway originally outlined by Raper (1928) has since been generally accepted though some modifications have been suggested (Fig. 2).

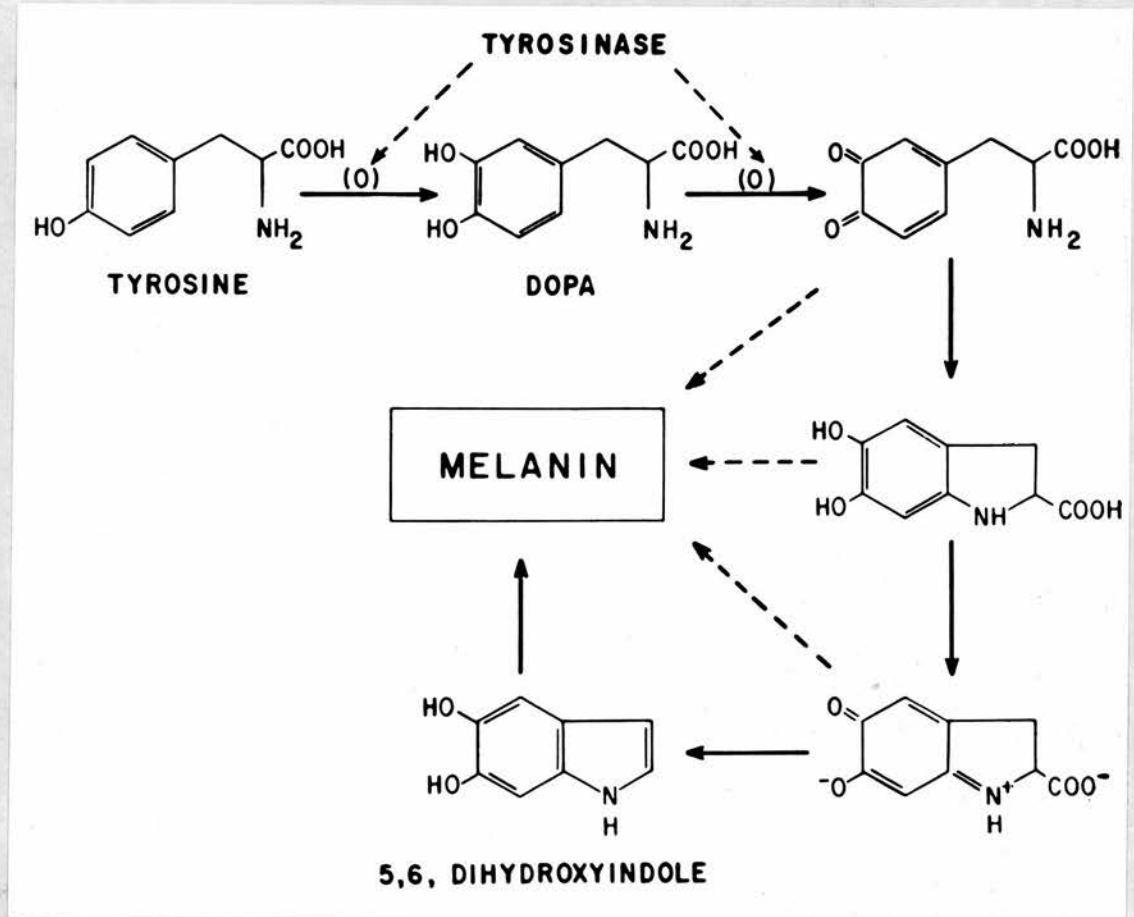


Figure 2: The tyrosine to melanin pathway.
(From Duchon et al., 1968)

The presence of tyrosinase is essential until the formation of dopaquinone. After this, further stages of the reaction proceed rapidly in the absence of the enzyme, though the reaction rates are increased in its presence. The indole-5, 6-quinone and other intermediates finally polymerise to melanin through polyindole quinone. Duchon et al. (1968) summarised

their views; "Vertebrate melanin is an insoluble, co-polymerizate of tyrosine intermediates, particularly 5, 6-dihydroxyindole, that are formed from tyrosine by the action of the copper-containing aerobic oxidase tyrosinase; the enzyme is confined to specialized cells, the melanocytes, and is usually attached to subcellular particles known as melanosomes."

According to Duchon et al. (1968), Nicolaus (1962), as a result of his extensive studies on melanin from the ink sac of cuttlefish (sepia), suggested a structure for melanin. It illustrates the alarming complexity of such polymerisation.

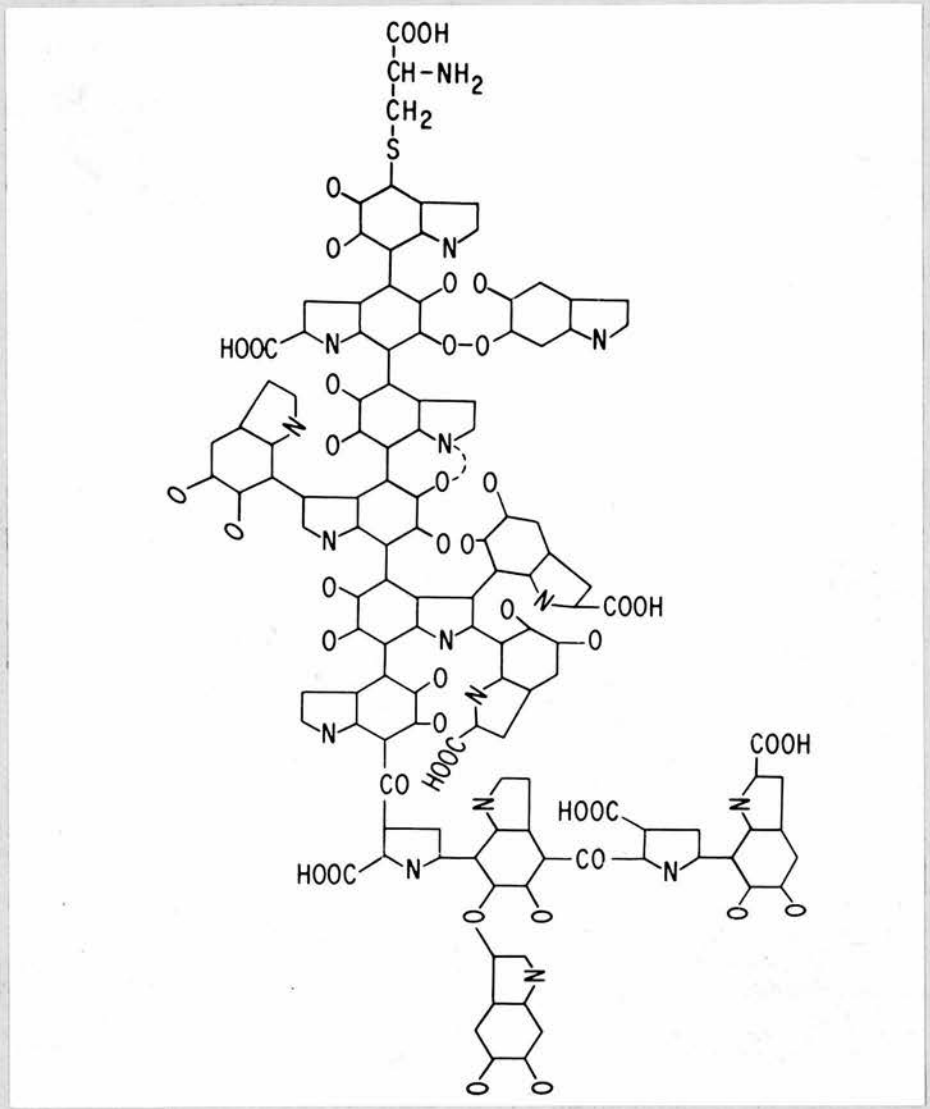


Figure 3: Nicolaus' scheme for the structure of sepia melanin. (From Duchon et al., 1968)

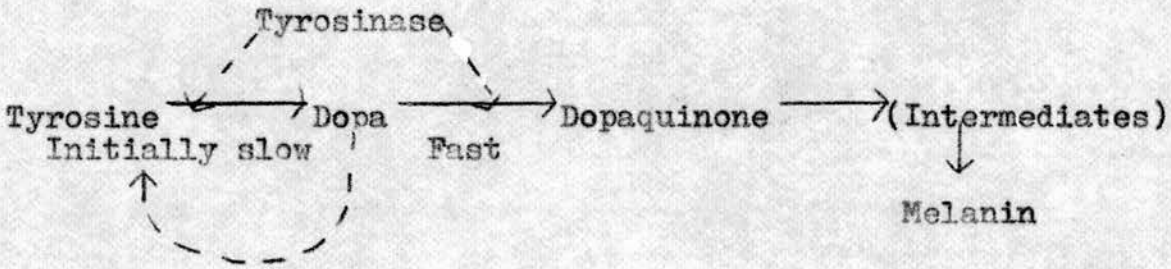
And so melanin (eumelanin), ranging in colour from brown to black, has a high molecular weight and a polymeric nature with a complicated irregular chemical structure. It is insoluble in almost all solvents and very resistant to most chemical treatments. Yellow and

red pigments in mammals (phaeomelanins) differ in their structure and are soluble in dilute alkalis. Prota and Nicolaus (1967) have suggested that phaeomelanins differ from eumelanins in that dopaquinone combines with cysteine to form 2-S and 5-S cysteinyl dopa and that further oxidation of these intermediates gives rise to phaeomelanins. Dopaquinone is therefore a common intermediate in the formation of both eumelanins and phaeomelanins.

Once melanin is formed within the cell it is bound to protein (melanoprotein). The linkages are very strong and can be broken only with drastic treatment such as prolonged acid hydrolysis at high temperature. According to Nicolaus and Piatelli (1965) the linkages are formed by sulphur-containing amino acids, especially cysteine, through a thio-ether linkage. The melanin polymer is also bound to the protein directly by peptide linkages between quinonoid intermediates of melanogenesis and the N terminus of polypeptide chains of the proteins.

Tyrosinases isolated from different sources have different characteristics (Lerner and Fitzpatrick, 1950). Mammalian tyrosinase occurs in various molecular forms (isotyrosinases) in the cell; two (T^1 + T^2) exist in soluble form and one (T^3) is bound to ribosomes, smooth surfaced membranes and melanosomes (Burnett et al., 1967). There is still debate as to whether tyrosinase catalyses both the hydroxylation of tyrosine

to dopa and the oxidation of dopa to dopaquinone (traditional view).



Okun et al. (1973a) have questioned this and believe that peroxidase rather than tyrosinase mediates the conversion of tyrosine to dopa and that tyrosinase functions only as a dopa oxidase as originally conceived by Bloch. This problem is considered in greater detail in pages 107-110.

The regulation of tyrosinase activity within the melanocyte is not known. Halprin and Ohkawara (1967) have produced evidence that reduced glutathione in the epidermis inhibits tyrosinase by combining with the copper present in the enzyme. They found decreased levels of glutathione reductase and reduced glutathione in negro skin and noted the photo-oxidising effect of ultraviolet on reduced glutathione. Their work has therefore uncovered the basis of a neat intraepidermal control mechanism of tyrosinase activity:

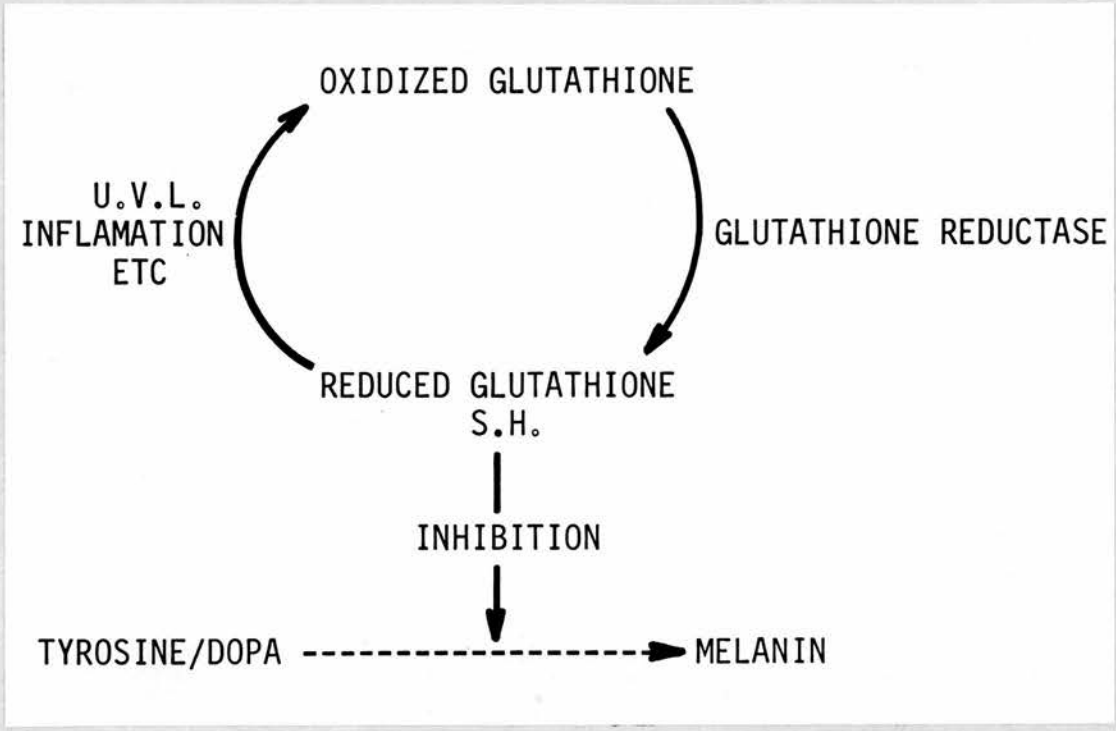


Figure 4: Role of glutathione in melanin synthesis. (From Halprin and Ohkawara, 1967)

Sulphydryl compounds might also inhibit melanin formation by forming complexes with the intermediates in the tyrosine ——→ melanin pathway (Roston, 1960 and Mason and Peterson, 1965). Burnett et al. (1967) considered that the soluble form of tyrosinase ($T^1 + T^2$) existed in the presence of such inhibitors until the enzyme became orientated or localised in such a way that it could carry out its specific function, (i.e. when enzyme bound to melanosome). This type of mechanism has been demonstrated by Chian and Wilgram (1967) during studies on a non pigmented mouse melanoma.

e. Subcellular sites of melanin biosynthesis

Painstaking and timely research as well as great technical advances in the late nineteen fifties allowed Seiji et al. (1961) to develop the melanosome concept - "melanin formation and deposition occur in cytoplasmic particles of the melanocyte because the enzyme tyrosinase is bound to specialized organelles, melanosomes, in the melanocyte." These researchers, working in Blaschko's laboratory in Oxford, carried out their studies on mouse melanoma. They measured tyrosinase and specific mitochondrial enzymes (cytochrome oxidase and succinic dehydrogenase) in electron microscopic monitored subcellular fractions separated by ultracentrifugation and density gradient centrifugation. The same techniques had been employed a few years earlier by Siekevitz and Palade (1958) in their classical studies on zymogen granules in exocrine cells of the pancreas.

The conclusions of Seiji and his colleagues were the subject of a vigorous controversy at the Fifth International Pigment Cell Conference in New York 1961. Dubuy and his co-workers (1963) felt that it was not possible to separate subcellular particles bearing succinic dehydrogenase and tyrosinase activity, and were unwilling to change their view that melanosomes were modified mitochondria. The debate has been discussed by Fitzpatrick et al. (1968) and is a good example of how disagreement can catalyse advancement

of scientific knowledge. After the Conference Fitzpatrick and his team repeated the experiments of Dubuy et al. using Dubuy's techniques. They discovered, with the help of electron microscopic monitoring, that Dubuy's separated subcellular fractions were not 'pure' and that mitochondria contaminated the melanosomal fraction. So small, but important, changes in technique then led to a common conclusion; and Seiji and Fitzpatrick's work has since been validated by other workers using different methods such as electron microscopic autoradiography. Nakai and Shubik (1964) have demonstrated that ^{14}C labelled dopa is incorporated in vitro into melanosomes and Moyer (1966), working with labelled tyrosine, has confirmed the in vitro incorporation of this precursor into melanosomes.

f. Ultrastructure of the melanocyte

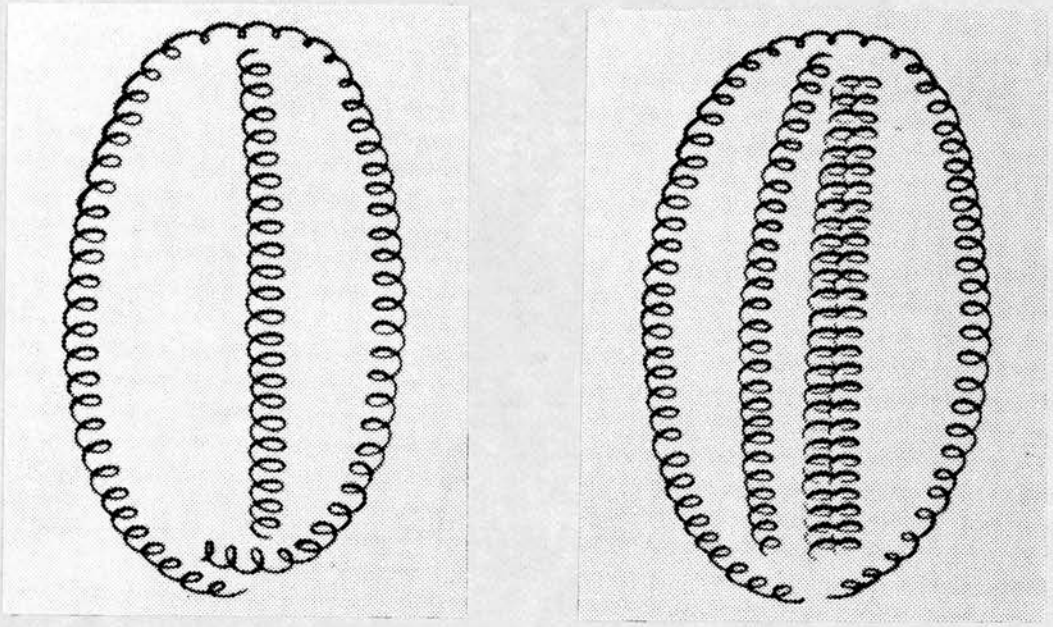
It is an invidious task to review electron microscopic work on the melanocyte carried out in the last thirty years. Knowledge has increased progressively, due more to technical advances than any startling new ideas. Only a few of the stepping stones which illustrate this have been chosen.

Mason et al. (1947) were the first to view pigment granules in human skin under the electron microscope. Their pictures showed rod shaped and globular granules, but no internal structure could be identified. Pease (1951) noted that the clear cells in the basal layer

of the epidermis did not contain tonofibrils. The pictures of human hair follicles presented by Birbeck et al. (1956) were of a higher quality and they were able to define more subcellular detail within the melanocyte. They noted that some pigment granules appeared to consist of several parallel lamellae forming cylindrical or ellipsoidal shells. They seemed to develop in vesicles situated in a region near the nucleus probably analagous to the Golgi region of other secretory cells. Odland (1958) confirmed that there were no tonofibrils in melanocytes and also noted that they were not attached to neighbouring cells by desmosomes. Charles and Ingram (1959) seem to have been the first to record the presence of regular transverse striations in some melanosomes in melanocytes of human skin, and this observation was confirmed a year later by Drochmans (1960). Zelickson and Hartmann (1961) stressed the differences between melanocytes and neighbouring keratinocytes and commented on the plentiful mitochondria and complex endoplasmic reticulum in melanocytes. Birbeck (1962) provided the best high resolution photograph (X160,000) of a melanosome to date.

Wellings and Siegel (1963) produced prints of human and mouse melanoma cells revealing even more detail. Their analysis enabled them to propose a scheme of melanin granule formation based on morphological evidence. They suggested that a tyrosinase-rich protein,

visible as minute particles, was synthesised at the ribosomes. These particles then accumulated in the endoplasmic reticulum and travelled to the Golgi apparatus where they were concentrated to form pre-melanin granules. The premelanin granules left the Golgi apparatus and were progressively melanized under the influence of tyrosinase to form large mature and dense melanin granules. In the same year Moyer (1963) came to similar conclusions after studying normal and malignant melanocytes in mice. In 1966, during a symposium on the Biology of Skin at Oregon, Drochmans (1967) showed high resolution pictures of melanosomes and deduced that coiled filaments made up part of the matrix. He considered that the first stage in formation of the melanosome was the deposition of a protein matrix composed of coiled filaments (Fig. 5a). Cross linking of these filaments both at the periphery and at the centre of the melanosome (Fig. 5b) was probably responsible for the transverse striations seen on micrographs. After formation of the protein matrix (possibly tyrosinase units or tyrosinase linked with a structural protein) melanin deposition gradually occurs, and the pigment accumulates on the inner membranes obscuring the characteristic periodicity (100A°) of the structures. Finally the organelle becomes a uniformly dense particle without discernable internal structure.



(a) (b)

Figure 5: Melanosomal formation
See text for explanation
(From Drochmans, 1967)

And so by the mid nineteen sixties there was a much clearer idea of the structure of the melanocyte and its organelles. Unfortunately the terminology was confused, but the terminology and classification of melanosomes now generally adopted is:

Melanosome

Definition (From Duchon et al., 1968)

"A melanosome is an organelle that is surrounded by a unit membrane and contains a highly organized internal

structure of longitudinally oriented strands or concentric lamellae that have a regular pattern of dense particles with a characteristic periodicity. The organelles may be spherical or ellipsoid and often contain tyrosinase."

Stages (From Fitzpatrick et al., 1971)

Stage I. A spherical, membrane-delineated vesicle may be called a melanosome if it

(i) is shown to contain tyrosinase by electron microscopy combined with histochemistry or

(ii) contains filaments that have a distinct periodicity of 100A°.

Stage II. The organelle is oval and shows numerous membranous filaments, with or without cross linking, having a distinct periodicity.

Stage III. The internal structure, characteristic of Stage II, has become partially obscured by electron-dense melanin.

Stage IV. The oval organelle is electron-opaque without discernable internal structure in routine preparations.

It would now seem probable that a point has been reached where further understanding of the ultrastructure

of the melanocyte will not depend on routine transmission electron microscopy. It is certainly difficult to imagine material superior to that published by Maul (1969), in his ultrastructural studies of human melanoma in vitro. His beautiful pictures confirm most of the observations described above, though his conclusions on the origin of melanosomes are, in some ways, different (see page 106). The events described above have been summarised diagramatically by Fitzpatrick et al. (1967).

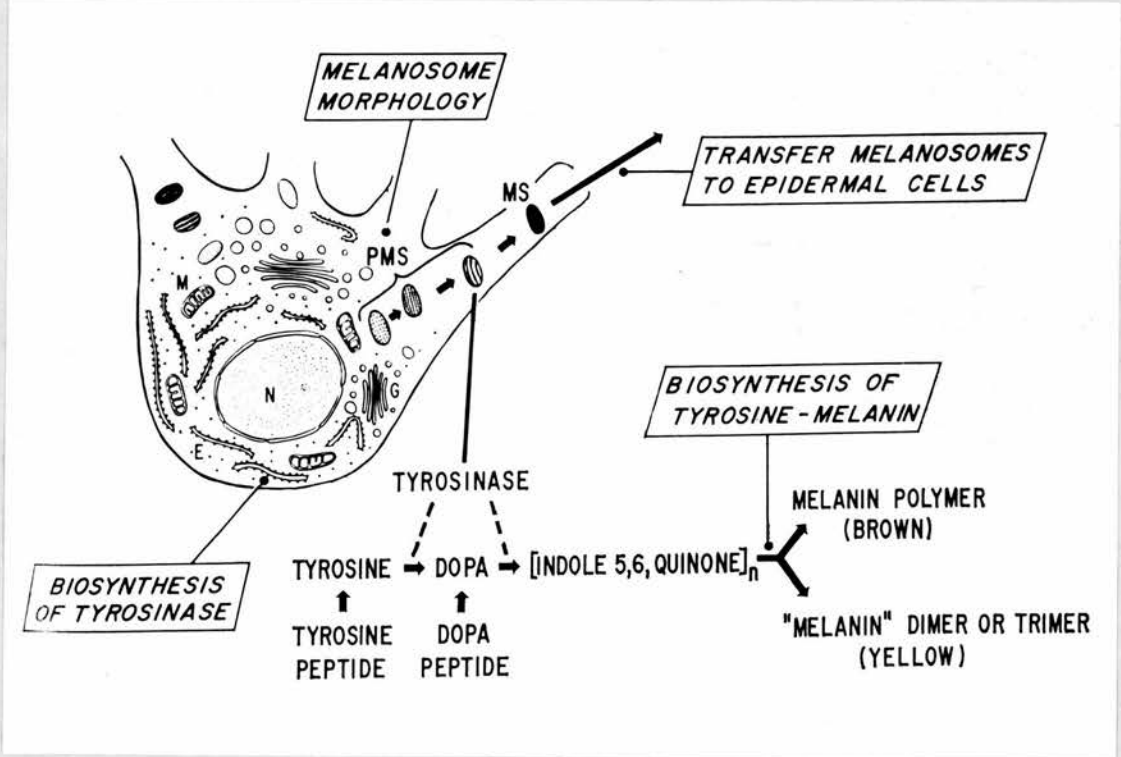


Figure 6: The melanocyte and melanin biosynthesis.
PMS = Stage I - III melanosomes. MS = Stage IV melanosome
(From Fitzpatrick et al., 1967)

g. Racial differences in pigmentation

It seems extraordinary that it was not until the late nineteen sixties that Man began to understand why negroes were black and caucasoids white. The work of Szabo and Wolff and their colleagues has clarified the problem and is worth summarising. As mentioned earlier there is no difference in the number of melanocytes between negroes and caucasoids (Billingham, 1949). There are however fewer melanosomes in the melanocytes and keratinocytes of caucasoids and mongoloids. Of those present in the melanocyte most are in Stages I, II and III. Those in the keratinocytes are in Stage IV but tend to be grouped in membrane-limited organelles to form "melanosome complexes". The appearance is different in negroids and Australian Aborigines. Here there are more melanosomes in the melanocytes and keratinocytes and a high proportion of melanosomes are seen at the IVth stage of development. Those in keratinocytes appear mostly on their own (Plate 7) rather than in complexes (Plate 5). Wolff and Konrad (Wolff and Konrad, 1972; Konrad and Wolff, 1973) have shown, both experimentally (using latex beads in guinea pig skin) and in human pigmentary disorders, that the complexing of melanosomes in keratinocytes is a size-dependent phenomenon. Particles of 0.1μ tended to be complexed whilst those with a diameter of 0.8μ were not.

In summary, it seems that the main reasons why negroids are black when compared with caucasoids are:

1) their melanocytes, though not increased in number, produce more melanosomes;
2) their melanosomes are more fully pigmented (i.e. more in Stage IV);
3) their melanosomes are larger;
4) their melanosomes are disposed individually in the keratinocytes.

(Szabo et al., 1969)

These are illustrated in Figure 7.

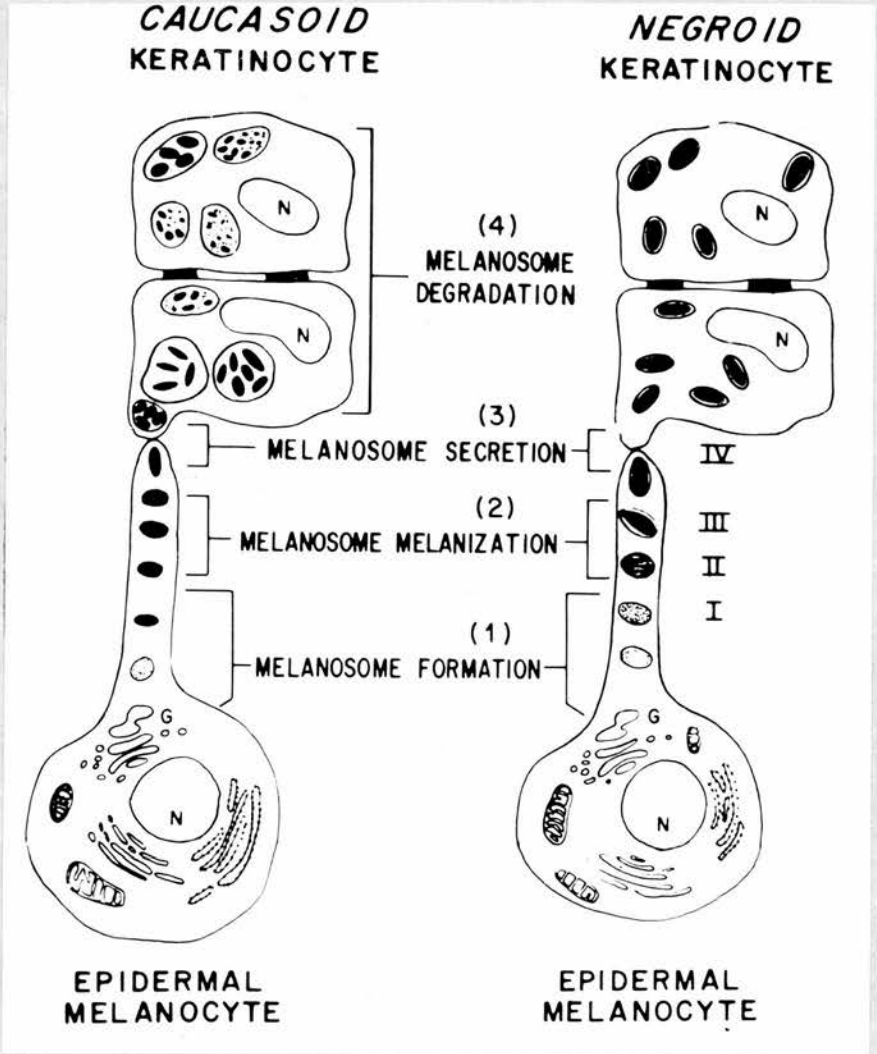


Figure 7: Diagrammatic representation of epidermal melanin units in caucasoids and negroids.
(From Toda et al., 1972)

It may be that, in caucasoids, melanosomes are degraded more rapidly in melanosomal complexes, as these are known to contain hydrolytic enzymes and can be regarded as lysosomes (Hori et al., 1968;

Wolff and Schreiner, 1970a; Wolff and Honigsmann, 1972).

It does not require much imagination to visualize how genetic factors could influence numerous points in the process of melanin production by the melanocyte, and melanosomal transfer to the keratinocyte. They could regulate early events in melanoblast migration, melanocyte morphology, melanosomal structure, tyrosinase activity, types of melanin synthesized, melanosomal transfer to keratinocytes and melanosomal degradation within keratinocytes (Quevedo, 1971).

3. THE LANGERHANS CELL

a. Paul Langerhans

Over one hundred years ago the German pathologist Paul Langerhans described a dendritic cell in human epidermis which stained with gold chloride (Langerhans, 1868). As the function of this cell remains unknown it continues to be named eponymously. For this reason, and also because the first personal contribution to this thesis concerns Paul Langerhans, it is worthwhile spending a little time outlining the life of this remarkable man. The biographical details are taken from a tribute to Paul Langerhans by Giacometti and Barss (1969).

Paul Langerhans was born in 1847 in Berlin. Whilst still a medical student working in Virchow's laboratory he published a paper describing high level intraepidermal dendritic cells in human skin. He demonstrated these using Cohnheim's gold chloride method (Cohnheim was one of his teachers), a procedure thought then to stain only nerves (Fig. 8).

Figure 8: Langerhans' original drawing of gold chloride staining cells in epidermis.
(From Langerhans, 1868)

Langerhans considered that the cells represented intraepidermal neural elements and that they had nothing to do with pigmentation. Although his original drawings depict a centrally directed process passing to the dermis, this has not been confirmed by later workers. Interestingly, Langerhans himself admitted that the

evidence for such a central process was rather slender.

The next year (1869), he completed his doctoral thesis for the University of Berlin on the "Microscopic Anatomy of the Abdominal Salivary Glands" and in so doing wrote his name into medical history. All doctors know of the islets in the pancreas, which he so clearly described in this thesis, and with which his name is invariably associated.

In 1870 he enlisted for medical duties in the Franco-Prussian war and saw service on the front. On his return he became Assistant Professor at the University of Freiberg and, in spite of a heavy teaching load, wrote papers on the histology of the myocardium and accessory glands of the genitals.

About this time he developed tuberculosis and eventually, because of his health, moved to the island of Madeira. Fortunately his health improved considerably and he spent thirteen busy and happy years there. He had a successful private practice, but still found time to publish important papers on polychaete worms (Ebling, 1974).

In 1885, when he was thirty eight he married and the happy couple had one child, a daughter. Alas, the marriage was short-lived; in July 1888 he succumbed to a renal disease, possibly related to tuberculosis. He was buried at the British Cemetery in Funchal.

In 1969 I read the fascinating tribute to Langerhans by Giacometti and Barss (1969). Luckily my wife and I were holidaying in Madeira in 1973. We visited the British Cemetery and, after a long search, my wife found the grave of Paul Langerhans, overgrown and all but hidden. We persuaded the gardener at the peaceful cemetery to tidy his resting place and returned the next day to find that he had kept his word (Plate 2).

Plate 2: The grave of Paul Langerhans.

The British Cemetery, Funchal, Madeira.

The epitaph from Homer's Odyssey was now clearly depicted: "My heart no longer wishes to go on living and behold the light of the sun." As Giacometti and Barss (1969) have pointed out this seemed a strange choice unless it was to express the grief of his wife. "Those who knew Langerhans spoke of him as a typical child of Berlin, of happy and genial disposition, with a keen desire to advance knowledge."

The story does not end here. Quite by chance sitting at the next dining room table in our hotel was a rather lonely looking man. We soon learned that he was German and recalled our visit to the grave of one of his expatriots. He seemed particularly interested in Langerhans and his work, and was appalled to hear that the grave of such a famous son of Germany should be so overlooked. Amazingly our acquaintance told us that he was a member of a National Committee, which, amongst other things, was responsible for the maintenance of graves of famous Germans. Without delay he would make the necessary arrangements with the German Consulate in Funchal to ensure that the grave of Langerhans would be looked after <u>in perpetuo</u>. Some months later we had a letter from our providential friend confirming that he had been successful (Pieper, 1973). By that time we had found out that our correspondent from Munich was himself a Christian philosopher of considerable fame, and the author of

numerous books, Professor Joseph Pieper.

Today the problems posed by the Langerhans cell are certainly more basic than those of the melanocyte. From the preceding sections it should be clear that considerable knowledge of the structure and function of the melanocyte has been gained over the last fifty years. Unfortunately, although much is known about the structure of the Langerhans cell little is understood about its origin and function. The problem has been discussed fully in some excellent reviews (Ferreira - Marques, 1951; Breathnach, 1965; Wolff, 1972 and Riley, 1975) and must surely now be reaching book proportions. The most significant findings will be detailed.

b. Histology and histochemistry of the Langerhans cell.

There is now no doubt that the Langerhans cell, high level clear cell (Masson, 1948) and white dendritic cell (Billingham, 1948) are identical. It is seen most commonly, though not exclusively, in the superficial part of the stratum Malpighii. Like the melanocyte the cytoplasm appears clear, and similarly, unless the tissue is fixed well, is apt to collapse around the convoluted nucleus leaving an empty space. At a light microscopic level the cell is never seen to contain pigment and has not been recognized in the horny layer. Time has shown that Billingham and Medawar (1953) were wrong in considering that melanocytes and Langerhans

cells either occur together in the epidermis or are both absent. Indeed either can be seen in the epidermis in the absence of the other.

Bloch's work on the melanocyte opened up numerous new avenues of research. The specific dopa reaction allowed counting of the cell in vertical sections and epidermal sheets, and much information about the melanocyte in normal skins and pathological conditions was obtained. Unfortunately no single histochemical technique for demonstration of the Langerhans cell in human skin has proved to be so dependable and specific. Gold impregnation, osmium iodide, quinone imine dyes, as well as methods for detecting nucleoside phosphatase, amino peptidase, α-D-mannosidase and other enzymes (see Riley, 1975) all have their advocates, but the number of different methods available tells its own tale of woe. The problem is not made easier by the fact that a suitable histochemical method for detecting the cell in one animal may be different from that in another (Wolff and Winkelmann, 1967). Nevertheless careful studies carried out with the necessary controls and, if possible, electron microscopic monitoring, are reliable (Wolff, 1972) and have led to important conclusions about the quantitative behaviour of the Langerhans cell population. Using the A.T.P.'ase method for detecting Langerhans cells in epidermal sheets of guinea pigs, Wolff and Winkelmann concluded that:

1) Langerhans cells contribute a surprisingly constant component of the epidermis (approximately 900 cells per mm^2);
2) there are no significant regional differences in Langerhans cell counts;
3) the number of Langerhans cells is independent of the type or intensity of pigmentation.

c. Ultrastructure of the Langerhans cell

Birbeck et al. (1961) were the first to outline the typical ultrastructural characteristics of the Langerhans cell.

1) No tonofibrils in cytoplasm. No desmosomes.
2) Indented nucleus.
3) Well developed Golgi apparatus.
4) No melanosomes.
5) Presence of a characteristic granule now referred to as "Langerhans cell granule".

The most specific of these is undoubtedly 5). There is now general agreement that cells containing typical granules can be called Langerhans cells. Unfortunately problems arise when criteria 1) - 4) are satisfied but not 5). This could be due to either a chance section through a Langerhans cell with few granules, or to the possibility that some cells may be immature Langerhans cells which do not contain any granules. Indeed serial sectioning has indicated that the latter type of cell

does occur. In any case such cells can at present only be termed "indeterminate" (Zelickson and Mottaz, 1968) (see page 59).

The cytoplasm and organelles of the Langerhans cell suggest that it is very active metabolically. There are numerous mitochondria, a well developed Golgi apparatus and endoplasmic reticulum, and often plentiful ribosomes. Lysosomes are seen frequently and may contain a granular matrix or whorled membranes (see Chapter III). However the most striking organelle is the Langerhans cell granule. This is disc shaped and often has a vesicle at one end, though occasionally one at both ends or in the middle. The most common image is a rod like profile with a central lamella showing cross striation with a periodicity of 60 - 90A^{o} (Wolff, 1972). Often, if the vesicle is at one end, a highly characteristic profile resembling a tennis racket is seen (Fig. 9). If the granules are cut face on a two-dimensional square lattice of particles may be apparent (Fig. 10).

From the analysis of different images obtained in serial sections, Wolff (1967) and Sagebiel and Reed (1968) have suggested that the granule is a flat plate-like structure with a spherical or hemispherical blob protruding from its surface. Variations include curved, twisted and cup like shapes of the disc (Fig. 9).

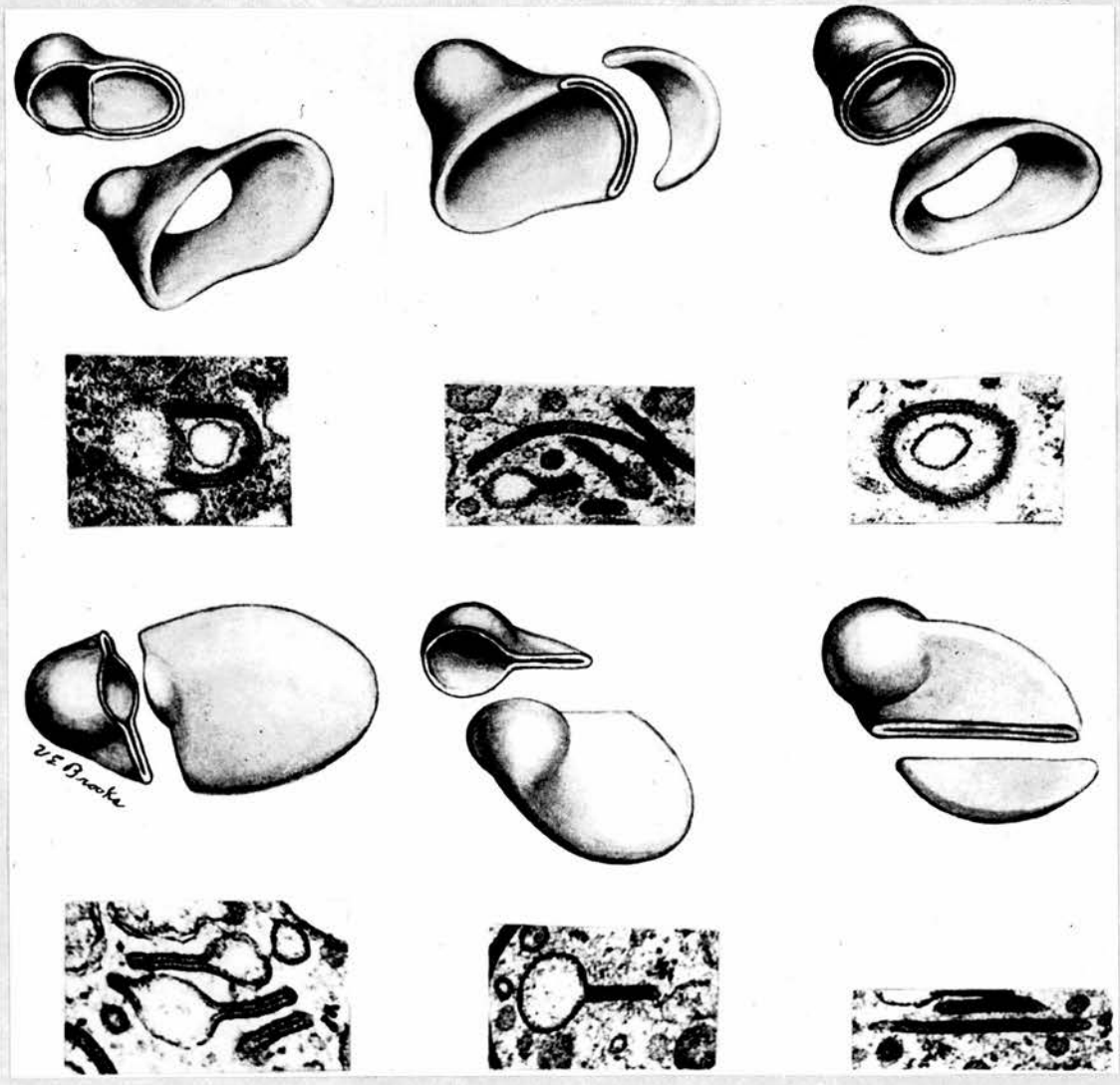


Figure 9: Three dimensional reconstruction of Langerhans cell granules.

(Figs. 9 and 10 from Sagebiel and Reed, 1968)

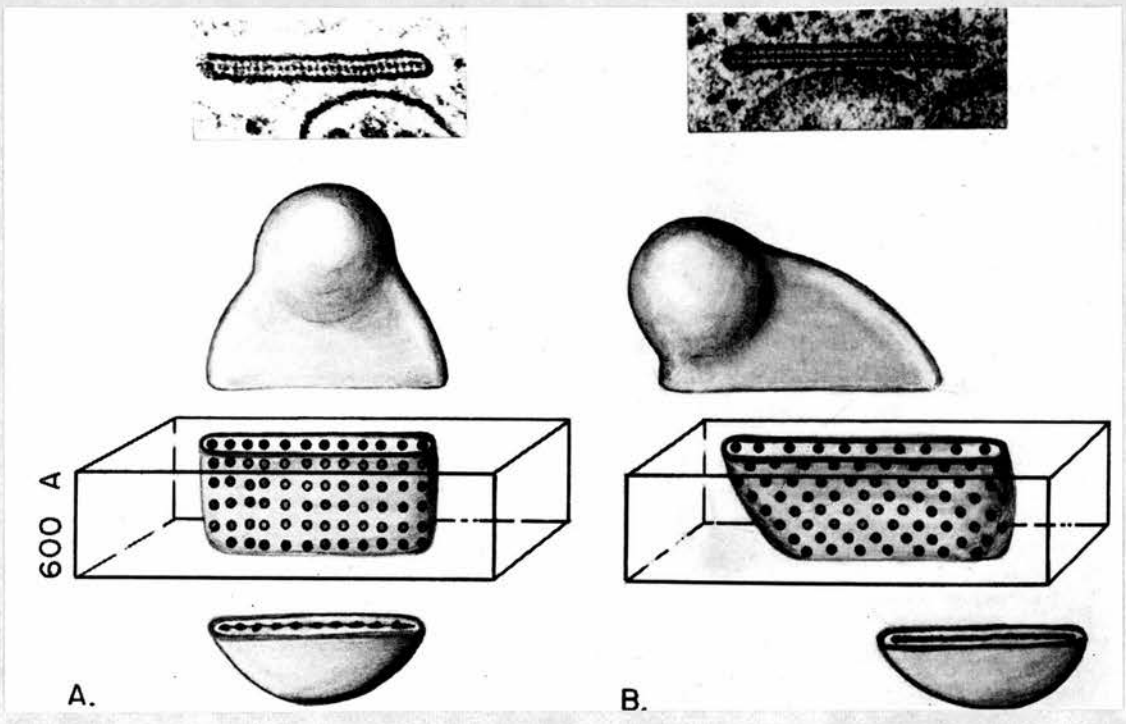


Figure 10: The appearance of the central lamella of the rod changes according to the angle of section.

A. Interrupted periodicity of 100A° (see Plates 17 and 18).

B. No periodicity evident (see Plates 19 and 20).

The granules are seen randomly distributed throughout the cytoplasm though some workers have mentioned greater numbers near the Golgi apparatus and rarely, in normal skin, they can be seen attached to the cell wall.

So far no enzymic activity has been detected within the granules using electron microscopic cytochemical techniques, though they stain with osmium zinc iodide (Niebauer et al., 1969), a reaction attributed to their high lipid content.

There is still no general agreement as to whether the Langerhans cell granules arise from the Golgi apparatus and travel to the cell membrane where they discharge their contents ("secretion theory" Breathnach, 1964), or whether they originate at the cell membrane by a process of endocytosis and migrate to the Golgi region ("endocytosis theory" Hashimoto, 1971). Points for and against both theories have been listed by Wolff (1972). Niebauer et al. (1969) mentioned that most researchers working on epidermal Langerhans cells prefer the secretion theory, whilst those investigating Histiocytosis X (see later) prefer the endocytosis hypothesis. Certainly experiments involving the uptake of tracer materials such as horse-radish peroxidase (Wolff and Schreiner, 1970), thorotrast (Wolff and Honigsmann, 1971) and ferritin (Sagebiel, 1972) lend no support to the endocytosis hypothesis.

d. The origin of the Langerhans cell

The earliest date at which Langerhans cells have been seen in human foetal epidermis is in the fourteenth week of gestation (Breathnach and Wyllie, 1965). At this time melanocytes are already present, and on this evidence alone it could not be denied that the Langerhans cell might be derived from the melanocyte. However the elegant experiments of Breathnach et al. (1968), in which neural crest-free skin grafts of mice were transplanted into spleens of histocompatible animals, revealed that after three to five weeks Langerhans cells were present in both the epidermis and dermis, whilst melanocytes, nerve axons and Schwann cells were consistently absent. This was thought to provide definitive evidence that melanocytes and Langerhans cells were of different lineage, and supported a mesodermal origin for the Langerhans cell. (The Langerhans cells noted in the epidermis could have come from the mesodermal cells in the subepidermal connective tissue of the graft or from the host spleen, either directly or from cells of the recipient circulating in the spleen.) Interestingly, with the exception of the thymus (initially of endodermal derivation, but later containing mesodermal components) all extraepithelial sites of Langerhans cells represent mesodermal compartments (Wolff, 1972).

Reams and Tompkins (1973) modified the above experiment and transplanted neural crest-free skin

grafts from mice into chick embryos. As Langerhans cells have not been found in the chick, their appearance in the grafted skin would mean that they had originated in the graft. Langerhans cells, but not melanocytes, were duly found in the graft and so these workers also concluded that the origin of the Langerhans cell and melanocyte was different. Two origins of the Langerhans cell seemed possible;

1) mesenchyme (subepidermal connective tissue) of graft;
2) epidermal cells (keratinocytes) of graft.

In an effort to find out which was most probable Reams and Tompkins then implanted mouse 'ectoderm' into normal chick skin and noted that the resulting chimeric epidermis contained Langerhans cells. They felt that this was good evidence for an ectodermal origin of the Langerhans cell and that the cell was derived from keratinocytes. Of course the results of their experiments depend on the assumption that Langerhans cells were not already present in the subepidermal connective tissue of grafts before transplantation, and that 'pure' ectodermal grafts (without adherent mesoderm) can be prepared. Both assumptions seem unjustifiable.

A thymic origin is unlikely because Langerhans cells have been found in the epidermis of athymic nude mice (Hunter et al., 1976).

Whatever the origin of Langerhans cells, it now

seems clear that, once they reach the epidermis, they form a stable self-replicating and constant cell population in which there is very little turnover of cells (Giacometti and Montagna, 1967; Schellander and Wolff, 1967; Wolff and Winkelmann, 1967 and Mackenzie, 1975).

e. Distribution of Langerhans cells in the body

The localization of cells which satisfy the ultrastructural criteria of Langerhans cells can best be described under

(i) epithelia capable of keratinisation;

(ii) other sites.

(i) Epithelia capable of keratinisation

Langerhans cells have been found in:

(a) normal epidermis (see Chapter III)

(b) epithelia of skin appendages, e.g. external root sheath of hair follicles (Breathnach, 1963), sebaceous glands (Jimbow et al., 1969a)

(c) oral, gingival and nasal mucosae (Schroeder and Theilade, 1966; Schenk, 1975)

(d) cervical mucosa (Hackermann et al., 1968)

(e) the mucosae of vitamin A deficient rat trachea and bladder (Wong and Buck, 1971)

(ii) Other sites

Langerhans cells are demonstrable in:

(a) dermis of normal skin (Zelickson, 1965)

(b) stroma of benign appendage tumours (Hashimoto and Tarnowski, 1968)

(c) inflammatory infiltrate in some skin conditions, e.g. pityriasis rosea (Hashimoto and Tarnowski, 1968)

(d) lymph nodes (Jimbow et al., 1969b; Shamoto et al., 1971)

(e) thymus (von Haelst, 1969)

(f) lesions of histiocytosis X, (Basset et al., 1965; Cancilla et al., 1967 and Morales et al., 1969).

f. Histiocytosis X

In 1965 Madame Basset and her associates noticed what they thought were viral inclusions in histiocytes of bony lesions of histiocytosis X. Soon it was realised that these inclusions were identical to Langerhans cell granules, and their observation changed the direction of research. Until that time most workers were thinking of some type of relationship between the Langerhans cell and the melanocyte, but the French findings immediately encouraged concentration on a histiocytic role for the Langerhans cell (Hashimoto, 1971). It is now known that the cell can be seen in all types of histiocytosis X, i.e. Letterer Siwe, Hand

Schüller Christian and eosinophilic granuloma types of the disease. Usually a considerable number, but not all, cells in the infiltrates of these conditions contain Langerhans cell granules, but histiocytes containing the granules are not necessarily seen in all lesions (Wolff, 1972).

Only minor structural variations in the granule have been noted in cells of histiocytosis X when compared with those in the epidermal Langerhans cell (Niebauer et al., 1969). The models reconstructed from three dimensional analysis of histiocytosis X granules (Tusques and Pradal, 1969) are very similar to those suggested by Sagebiel and Reed (1968) for epidermal Langerhans cells and it seems probable that histiocytosis X represents some disorder, either reactive or neoplastic, of the Langerhans cell.

As histiocytosis X cells can be cultured in large numbers <u>in vitro</u> more easily than epidermal Langerhans cells, this allowed Basset and her colleagues to carry out studies similar to those which Seiji et al. (1961) had used when investigating the melanosome. Subcellular fractions were obtained in which the granules appeared mixed with microsomes and membranes. Enzymatic activities have so far not been detected in the granules but they are disrupted by treatment with pronase and trypsin. R.N.A.'ase reduces the density of the central lamella and the internal leaflet of the membrane and causes the whole

granule to swell. Hyaluronidase and D.N.A.'ase have no effect (Basset et Nezelof, 1969; Nezelof et al., 1973).

g. Function of the Langerhans cell

Today the function of the Langerhans cell still remains a matter of conjecture and the problem must be more perplexing than Langerhans could have ever dreamed. Neural theories gave way to ideas that the cell was, in some way, related to the melanocyte. The discovery of the cells in histiocytosis X led to views that it was a type of phagocyte. Still the jigsaw did not fit and ideas ranging from proposals that the cell influences keratinisation to notions that it is some form of immunocyte indicate the complexity of the problem.

At a meeting of the Scottish Dermatological Society on March 9th, 1972 I reviewed the problem of the function of the cell and summarised my interpretation of recent publications (Hunter, 1972b). The following is an updated version of my 1972 paper.

When considering the function of the Langerhans cell four broad possibilities have attracted interest.

(i) The cell is a functioning neural element.

(ii) The cell is related to the melanocyte system.

(iii) The cell is a specialised macrophage.

(iv) The cell influences keratinisation.

(i) <u>The Langerhans cell as a functioning neural element</u>

Langerhans cells may be:

modified Schwann cells which have migrated from the dermis into the epidermis (Ferreira-Marques, 1951)

an intraepidermal component of peripheral autonomic nervous system (Richter, 1956)

part of a 'neurohormonal' system of the skin, (Wiedmann, 1952).

<u>For</u>:

Langerhans cells stain similarly to nerve cells with gold chloride, methylene blue and osmium iodide (Langerhans, 1868; Mishima and Miller-Malinska, 1961; Niebauer et al., 1969). A.T.P.'ase reaction also similar.

Light microscopic histochemical methods reveal possible connections with papillary dermal plexus of nerves and a decrease in number of Langerhans cells when there is nerve destruction (e.g. Leprosy, Richter, 1956).

<u>Against</u>:

Most staining techniques capricious. Light microscopy does not reveal connections between Langerhans cells (A.T.P.'ase) and dermal nerve endings (Winkelmann, 1960).

Dermal nerve fibres are not seen crossing basal lamina in normal skin. Electron microscopic appearance

unlike nerves or glial cells. Electron microscopy reveals that Schwann cells are only rarely seen in mitosis and are not related to the dendrites of Langerhans cells (Breathnach, 1965). Electron microscopic evidence for presence of Langerhans cells when dermal nerve fibre destruction in Leprosy (Breathnach et al., 1962).

Conclusion

The Langerhans cell is not a functioning neural element.

(ii) The Langerhans cell is related to the melanocyte system

Langerhans cells may represent effete melanocytes (Masson, 1951; Billingham and Medawar, 1953).

Langerhans cells are division products of melanocytes (Masson, 1951; Breathnach, 1963).

Melanocytes and Langerhans cells are derived from same stem cell (Zelickson, 1967).

For:

Similar morphology (dendritic, no desmosomes and no tonofibrils).

Roughly a 1:1 ratio between numbers and distribution similar (Billingham and Medawar, 1953).

Reciprocal relationship between melanocytes and Langerhans cells in guinea pigs when exposed to ultra-violet irradiation, Thorium X and X-rays (Fan et al., 1959).

Reciprocal relationship between melanocytes and Langerhans cells in humans in vitiligo and following ultraviolet irradiation - light and electron microscopy (Birbeck et al., 1961; Zelickson and Mottaz, 1970).

Reciprocal relationship between melanocytes and Langerhans cells in cultures of guinea pig epidermal cells (Cruikshank and Cooper, 1975).

Melanocytes and Langerhans cells visible in epidermis of fourteen week human foetus (Breathnach and Wyllie, 1965).

Against:

Electron microscopic structure is not that of an effete cell but is suggestive of one actively synthesising protein and granules.

In guinea pig, no fixed ratio between epidermal A.T.P.'ase and dopa positive cells at different sites (Wolff and Winkelmann, 1967).

In guinea pig, increase in dopa positive cells but no change in number of A.T.P.'ase positive cells after ultraviolet stimulation (Wolff and Winkelmann, 1967).

Vitiligo: no increase in A.T.P.'ase positive cells (Brown et al., 1967).

Carcinogenic substances applied to Syrian hamsters cause melanomas but no increase in Langerhans cells (Rappaport et al., 1963).

Depigmenting agents damage melanocytes but not

Langerhans cells (Bleehen et al., 1968).

Langerhans cells capable of mitosis (Giacometti and Montagna, 1967; Mackenzie, 1975).

Langerhans cells appear in neural crest-free explants of mouse foetal skin onto spleen (Silvers, 1957; Breathnach et al., 1968) and into chick (Reams and Tompkins, 1973).

Langerhans cells present in duct of sebaceous glands where there are no melanocytes (Breathnach, 1965).

Melanocytes rarely contain Langerhans granules (Mottaz et al., 1971).

Langerhans cells rarely contain melanosomes (Mishima, 1966; Zelickson, 1965).

Langerhans cells - electron microscopic dopa reaction negative (Hunter et al., 1968).

Conclusion

It seems unlikely that there is any direct relationship between melanocytes and Langerhans cells. It may be that both populations of cells share similar territories, particularly in the basal layer of the epidermis, and that some of the above findings can be explained on the basis of mutual territorial competition (Breathnach et al., 1968 and Riley, 1975).

(iii) The Langerhans cell is a specialised macrophage.

Langerhans cells are a type of intra-epidermal macrophage and, in particular, may behave like

macrophages in cell mediated immune reactions or have some other local immunological function.

For:

Morphologically similar to macrophage, though latter does not usually contain Langerhans granules (Tarnowski and Hashimoto, 1967).

Occurs in histiocytic tumours (Basset et al., 1965; Cancilla, 1967; Hashimoto and Tarnowski, 1968).

Lysosomal/hydrolytic enzyme content similar (Wolff and Winkelmann, 1967; Tarnowski and Hashimoto, 1967).

Phagocytic potential. Can engulf:

melanosomes (Zelickson, 1965; Breathnach and Wyllie, 1965; Mishima, 1966);

ferritin (Nordquist et al., 1966);

horse-radish peroxidase (Hashimoto, 1971).

Present in sites suggesting mesenchymal origin:

stroma of skin appendages (Hashimoto and Tarnowski, 1968);

lymph nodes (Jimbow et al., 1969).

Apposition of Langerhans cells to lymphocytes noted in epidermis after experimental production of contact dermatitis, but not seen in primary irritant reactions (Silberberg et al., 1971, 1973 and 1974). Silberberg and his colleagues (1975) have also noted apposition of Langerhans cells to effector lymphocytes in the dermis of passively induced contact dermatitis. The following interpretation has been proposed by Silberberg

et al., (1975):

> "Some of the mononuclear cells apposed to Langerhans cells are specifically sensitized lymphocytes which interact with antigens on or near the surface of Langerhans cells. In response to factors, lymphokines, which are released when sensitized lymphocytes contact antigen, damage to some Langerhans cells, as evidenced by ultrastructural changes, occurs. Thus Langerhans cells may be targets for the specifically sensitized lymphocytes, with substances released from lysosomes and other organelles of the Langerhans cells, leading to further inflammatory changes."

Against:

Macrophages rarely contain Langerhans cell granules.

Keratinocytes engulf horse-radish peroxidase more avidly than Langerhans cells. Horse-radish peroxidase seen only in Langerhans cell granules attached to cell wall. Tubular epithelium of kidney and hepatocytes have similar non specific phagocytic activity (Wolff and Schreiner, 1970).

Only few of the enzyme activities demonstrated in Langerhans cells are characteristic of lysosomal enzymes usually associated with macrophages (Riley, 1975).

Behaviour of Langerhans cells in vitro is entirely different from that of known macrophages (Cruikshank and

Cooper, 1975).

Conclusion

Its low phagocytic potential rules out a role as a conventional macrophage (as defined by Langervoort et al., 1970) but a function involving interaction with antigen, or other forms of participation in cell mediated reactions in the skin, is possible. However it is not a modified T lymphocyte because:

a) Langerhans cells remain present in mouse epidermis when animals given anti-lymphocytic serum and immuno-suppressing agents (Reams and Greco, 1969 and 1970);

b) Langerhans cells appear normal in the epidermis of the athymic nu/nu mouse (Hunter et al., 1975). These animals are T lymphocyte deficient; delayed hypersensitivity reactions, including contact dermatitis, are abolished and grafts of foreign tissue are accepted (Cubie, 1976).

(iv) Relationship with keratinisation

Langerhans cells may influence the orderly process of orthokeratotic keratinisation, or regulate epidermal cell turnover.

For:

Langerhans cells found in other epithelia capable of keratinisation:

human cervix (Hackemann et al., 1971)

vitamin A-deficient rat trachea and bladder (Wong and Buck, 1971).

Increased number of Langerhans cells in the more heavily keratinised (slow turnover rate) oral mucosa compared with non keratinised mucosa (Hutchens et al., 1971; Sagebiel et al., 1971).

Increased number of Langerhans cells in ichthyosis with hyperkeratosis (Giacometti, 1968) but decreased in psoriasis with parakeratosis (Giacometti, 1968; Szekeres, 1970).

Presence of Langerhans cells in the lesions of benign hyperkeratotic tumours such as viral warts (Fritsch, 1971) and seborrhoeic warts (Molokhia and Portnoy, 1971).

Relationship between Langerhans cells and stacking of the horny layer cells in mouse epidermis (Mackenzie, 1972; Allen and Potten, 1974).

Distribution in mouse tail related to a special form of keratinisation, associated with a granular layer and a basket weave type of keratin (Riley, 1966).

There is an inverse relationship between the number of Langerhans cells and epidermal mitotic rate (Sagebiel et al., 1971).

Removal of keratin by sellotape stripping results in:

a) initial decrease in A.T.P.'ase positive cells (Lessard et al., 1966 and 1968);

b) appearance of parts of Langerhans cells in

keratinocytes and melanocytes (Mottaz et al., 1971);

c) reconstitution of the epithelium before Langerhans cell repopulation (Lessard et al., 1968).

Langerhans cells migrate with epidermal cells during re-epithelialization in wound healing (Giacometti, 1969).

In vitro studies show a close relationship with keratinocytes (Constable, 1975) and Langerhans cells appear to act as 'organizers' (Cruikshank and Cooper, 1975).

Against:

After tape stripping of epidermis it takes about 15 days for the number of Langerhans cells to return to normal, but only 4 days for the epidermis to resume its normal keratinizing function (Lessard et al., 1968).

Langerhans cells are not found in Hassal's bodies of the thymus (van Haelst, 1969).

A higher number of Langerhans cells are found in the thickened epidermis of the mouse footpad (Mackenzie and Squier, 1975) when compared with the thinned epidermis of the mouse ear (Mackenzie, 1972) even though the labelling index and rate of regeneration of mouse footpad epidermis is higher than that of ear epidermis (Christophers and Laurence, 1973; Mackenzie, 1974).

Conclusion

Although Langerhans cells are certainly not responsible

for the process of keratinisation per se, it seems probable that they are involved in regulating the process, perhaps by maintaining a physiological equilibrium between differentiation and mitotic activity (Prunieras, 1969).

Cruickshank's 'organizing' theory is not only attractive, but is supported by impressive time lapse cinematographic evidence in vitro (personal viewing of film, 1973; Cruickshank and Cooper, 1975). The hypothesis of an "Epidermal Langerhans Cell Unit" (Wolff and Winkelmann, 1967a), consisting of the Langerhans cell surrounded by a clone of keratinocytes, all functioning as a unit, seems justifiable.

4. THE INDETERMINATE DENDRITIC CELL

The epidermis contains a third dendritic cell (Breathnach et al., 1963) which has been described under numerous different names ("Type-3 cells", Snell, 1965; "Indeterminate" cells, Zelickson and Mottaz, 1968; "Alpha" cells, Mishima et al., 1972). Its identification is best made at an ultrastructural level as it lacks tonofibrils and desmosomal attachments with surrounding cells. It has cytoplasmic features similar to those of the melanocyte and Langerhans cell except that it contains no melanosomes or Langerhans cell granules. It is usually found in the basal and suprabasal layer of the epidermis (Zelickson and Mottaz, 1968).

The indeterminate cell could be an immature precursor of the melanocyte or Langerhans cell (Reams and Tompkins, 1973). It could also be either a melanocyte or Langerhans cell with so few melanosomes or Langerhans cell granules that none are seen on thin sectioning. Wolff (1972) has summarised the relevant evidence, but no firm conclusions can be drawn, and they will not be discussed further in this study.

5. INTRODUCTION TO THE PRESENT WORK

The present work was started in 1968 during a visit to the electron microscopic laboratory of Dr. A.S. Zelickson in Minneapolis, U.S.A. Dr. Zelickson's interest in epidermal dendritic cells soon became infective, and my studies on them have continued. This thesis describes and correlates these studies.

Ultrastructural change in human epidermal dendritic cells is the central theme of this study. It has been produced by artificial and measurable forms of stress (ultraviolet irradiation, suction and friction) and studied in pathological conditions where the nature of the stress is ill-understood (malignant transformation). Fine structural changes in melanocytes and Langerhans cells are compared and contrasted, deductions concerning the structure and function of these cells made, and views on the histogenesis of malignant melanoma and histiocytosis X proposed.

When different avenues of research are explored there are inevitably some blind alleys as well as previously unsuspected bonuses. The studies described here are no exception. Perhaps the greatest regret, concerning these studies, is that a form of stress causing an alteration in keratinisation was not chosen, in view of the possible role of the Langerhans cell in this process (page 55). However studies investigating this and possible immunological functions of the

Langerhans cell are underway at present (e.g. Hunter et al., 1976); so the direction of research on this cell has certainly altered.

Chapter II

MATERIALS AND METHODS

Including

1. LIGHT MICROSCOPY

2. ELECTRON MICROSCOPY

3. PHOTOGRAPHY

4. OTHER METHODS

1. LIGHT MICROSCOPY

Tissue was fixed in Bouin's fluid or formalin, dehydrated, embedded in paraffin wax, sectioned and stained in a routine pathological laboratory. The staining methods were standard.

Routine - haematoxylin and eosin (H and E) (Drury and Wallington, 1967).

Basement membrane - periodic acid - Schiff (PAS) (Drury and Wallington, 1967).

Melanin - Masson Fontana (Drury and Wallington, 1967).

Some semi thin (1μm) epoxy resin embedded sections were studied. Stains used were toluidine blue (Kay, 1965) and haematoxylin and eosin (Chang, 1972)

2. ELECTRON MICROSCOPY

a. Fixation

The following fixatives were used routinely:

(i) 3% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 (Chapters III, IV, V, VI and VII) 5 - 24 hours at 4°C;

(ii) 2.5% glutaraldehyde + 1% paraformaldehyde in 0.1M cacodylate buffer (Karnovsky, 1965) (Chapters III, V, VI, VIII and IX) 5 hours at room temperature

In nearly all experiments the fixative was made up fresh (i.e. within 12 hours of experiment).

(iii) Osmic acid was used for post fixation in all experiments. Initially 1% OSO_4 in collidine buffer was used (Chapters III, IV, V and VI) but recently 3% aqueous OSO_4 was found to be more satisfactory (Chapters VII, VIII and IX).

b. Embedding media

Epoxy resins were used.

(i) Epon 812 (Luft, 1961) (Chapters III and IV).

(ii) Araldite (Glauert and Glauert, 1958) (Chapters III, V, VI, VII, VIII and IX)

c. Staining

In most experiments (but not in Chapter IV) the blocks were precontrasted with 0.5% uranyl acetate in

0.8M veronal acetate buffer or water (Farquhar and Palade, 1965) after postfixation in OSO_4.

Sections mounted on grids were stained with a freshly prepared saturated solution of uranyl acetate in 50% alcohol, then thoroughly rinsed and poststained in lead citrate (Reynolds, 1963).

d. Routine schedule

There were only minor variations in the following schedule.

Tissue, obtained either after surgical excision (general anaesthetic) or as a biopsy performed with or without local anaesthetic.

Immediate immersion in fixative.

One minute later specimen diced into cubes of about $0.5mm^3$ under drop of fixative on dental wax.

Fixation, 5 - 24 hours (room temperature or 4^oC).

Rinse X 3. Each 20 minutes. 0.1M cacodylate or phosphate buffer. 4^oC.

Storage (usually only overnight) in buffer $\pm$ added sucrose (Weakley, 1972). 4^oC.

Post fixation. 1 - 2 hours OSO_4. Icebath

Precontrasting in uranyl acetate. 45 minutes. 4^oC + in dark.

50% ethyl alcohol. 5 minutes.

70% ethyl alcohol. 5 minutes X 2.

95% ethyl alcohol. 5 minutes X 2.

Absolute ethyl alcohol X 3.

20 minutes fridge

20 minutes 4°C initially but increasing to room temperature

20 minutes room temperature.

Propylene oxide, 2:1, plastic - 1 hour - room temperature

Propylene oxide, 1:1, plastic - 3 hours - room temperature

Propylene oxide, 1:2, plastic - overnight - room temperature

Impregnation in plastic mixture - 24 hours - room temperature (in trough - in desiccator)

Embedding and orientation in capsules or moulds using fresh plastic mixture.

Polymerisation. 1 - 3 days at 60°C.

N.B. Neat propylene oxide was used (15 minutes X 2) after the absolute alcohol stage in the early experiments only (Chapters III and IV)

e. Thin sectioning

LKB II and LKB III ultratomes were used.

f. Electron microscopes

An R.C.A. E.M.U.-3 (Chapter IV), an AEI E.M.6 (Chapters V and VI) and an AEI Corinth 275 (Chapters III, VII and VIII) were used.

g. Magnification and measurement of size

Machine magnification was regularly checked using a calibration grid (replica of a diffraction grating in

which the distance between the parallel lines is accurately known). Size was then measured using an ultrastructure size calculator (Polaron Equipment Ltd.).

3. PHOTOGRAPHY

Standard methods were employed.

A Devere 54 Varicon enlarger with a point source was used and most prints were obtained with an Ilford rapid print processing machine (Super 12, Mark II).

4. OTHER METHODS

More specialized methods will be outlined at the beginning of each experiment.

Chapter III

NORMAL EPIDERMIS

Including

1. INTRODUCTION

2. MATERIALS AND METHODS

3. RESULTS
   - a. Light Microscopy
   - b. Electron Microscopy
     - (1) The keratinocyte and horny cell
     - (2) The melanocyte
     - (3) The Langerhans cell

4. DISCUSSION

1. INTRODUCTION

It is, of course, essential to have a thorough knowledge of the ultrastructure of normal dendritic cells before calling any appearance abnormal. Within small, but sometimes appreciable, limits the appearance of their fine structure will vary according to the processing and staining techniques employed, and for this reason it is important to establish a norm for the routine schedule of any laboratory.

The aim of the study in this chapter was therefore to define the ultrastructural appearance of normal epidermis using the same techniques as in later experiments.

2. MATERIAL AND METHODS

Normal skin was obtained from volunteers. Initially small elliptical biopsies were taken under local anaesthesia (1% xylocaine), but recently shave biopsies have been carried out with and without local anaesthesia. Over twenty biopsies were examined, most being taken from the forearm or lower back.

3. RESULTS

a. Light microscopy

Semi-thin plastic embedded sections reveal detail superior to that seen in routine paraffin embedded material. With practice the epidermal dendritic cells can be picked out with relative ease and thin sections cut to include them. Plate 1 shows normal epidermis and all cell layers are distinct. Probable melanocytes in the basal layer are arrowed (black).

The white arrows point to mid epidermal cells different in character from the surrounding keratinocytes of the squamous cell layer. The nuclei are convoluted and the cytoplasm less dense. No prickles (desmosomes) are seen connecting them to neighbouring cells. These are most probably Langerhans cells.

However at the light microscopic level it is impossible to be absolutely certain of the type of cells which are not keratinocytes. If they are in the basal layer then they are most likely melanocytes and if higher up in the epidermis, Langerhans cells. But melanocytes above the basal layer and Langerhans cells on the basal layer (e.g. Plate 15) can be seen, so the epidermal situation of these cells is not a reliable pointer to their character. The occasional mononuclear inflammatory cell can also be seen infiltrating normal skin and, at a light microscopic level, these cannot be distinguished from dendritic cells. Finally electron

microscopy is needed to distinguish dendritic cells from 'indeterminate' cells (see Chapter I.4)

b. Electron microscopy

(1) The keratinocyte and horny cell

The bulk of the epidermis is occupied by keratinocytes and cells of the horny layer. Keratinocytes are distinctive in that they contain small fibres (tonofilaments) aggregated into bundles, called tonofibrils, and are attached to each other by desmosomes (Plate 3). Free ribosomes are usually plentiful but mitochondria are sparse and the Golgi apparatus poorly developed. Melanosomes in the cytoplasm of keratinocytes are seen either disposed individually or complexed in groups (Plates 5 and 7).

The cell surface consists of numerous interdigitatory processes which are attached to those of neighbouring cells by desmosomes (Plates 3 and 4).

In the basal layer the keratinocytes are aligned vertically on the basal lamina to which they are attached by hemidesmosomes (Plate 7). In mid epidermis they appear polygonal but become flattened as they reach the level of the granular cell layer. Here orderly destruction of cell contents occurs, and their nuclei, mitochondria and other organelles become degenerate (Plate 8). At the same level keratohyalin granules and membrane coating granules appear in the cell (Plate 8) and the cell wall becomes thickened.

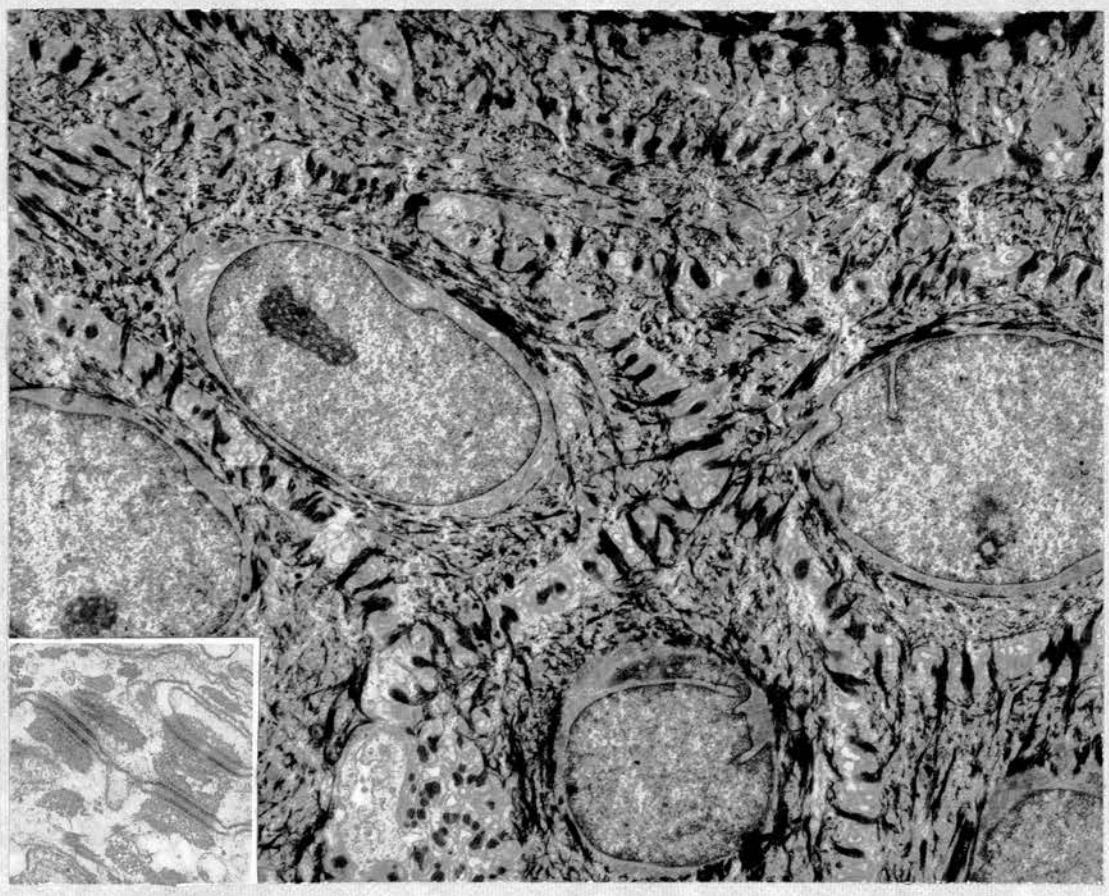


Plate 3 (X 6,000):

Keratinocytes in squamous cell layer. Granular cell layer top right. Note numerous tonofibrils in cells and desmosomes connecting cells. Inset (X 32,500) shows 3 desmosomes.

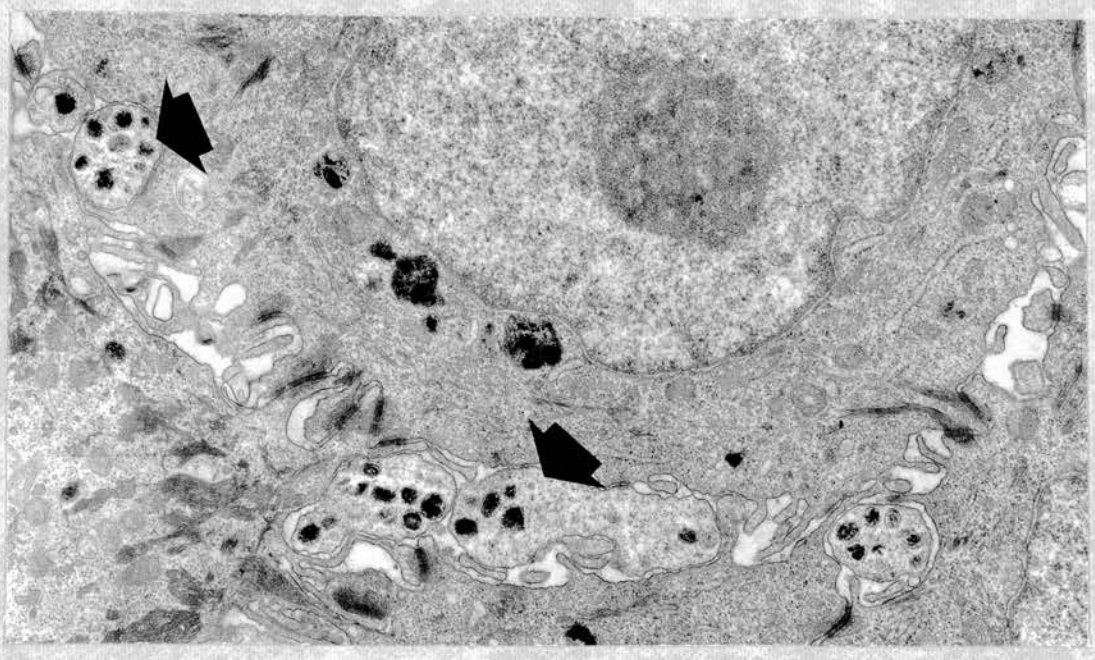


Plate 4 (X 14,000): Keratinocytes with dendritic processes of melanocytes. Dendritic processes (arrows), in intercellular space, contain mostly Stage IV melanosomes.

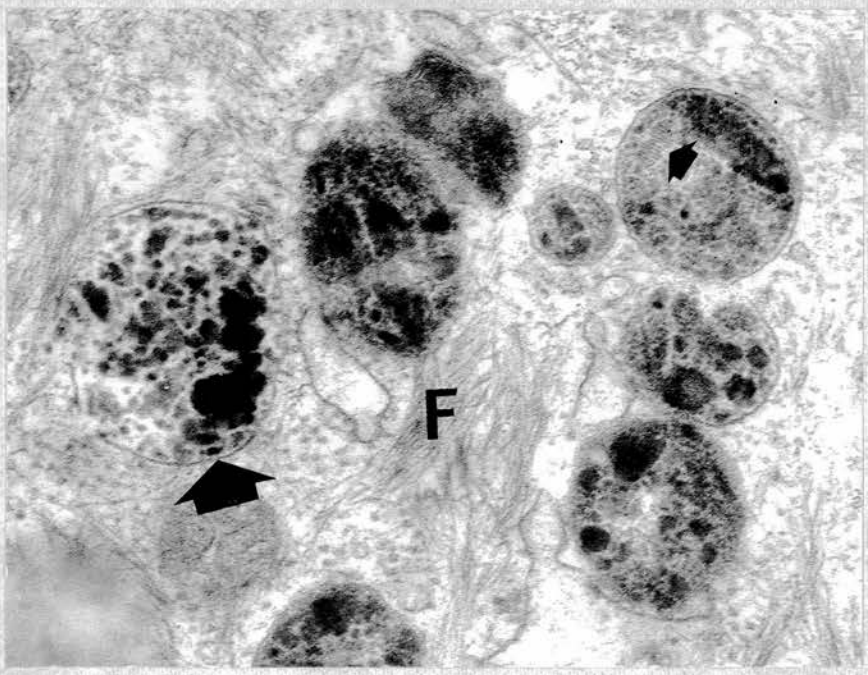


Plate 5 (X 59,000): Melanosome complexes in keratinocyte. They are bound by a unit membrane (large arrow) and periodicity in some remnants can be seen (small arrow) F, tonofibrils.

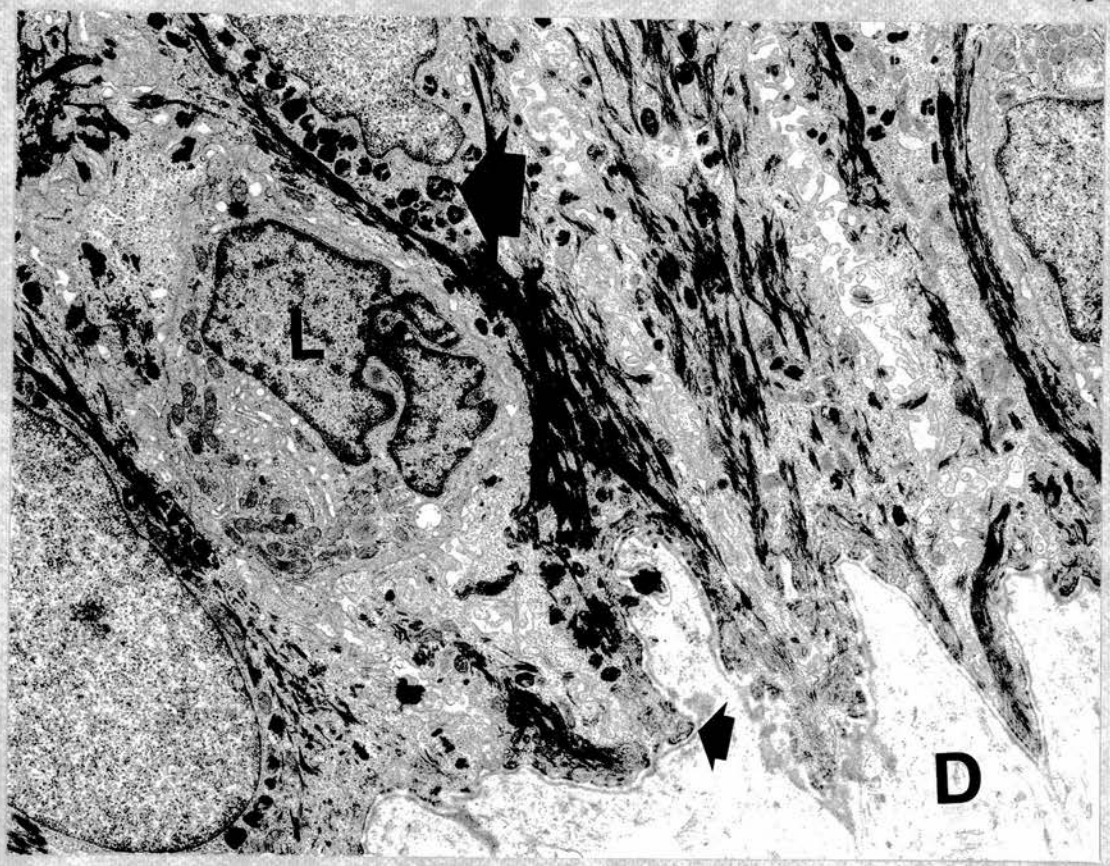


Plate 6 (X 7,500): Basal cell layer (tanned Caucasoid) Note most of melanosomes are in complexes (large arrow) L, Langerhans cell; D, dermis; small arrow points to basal lamina.

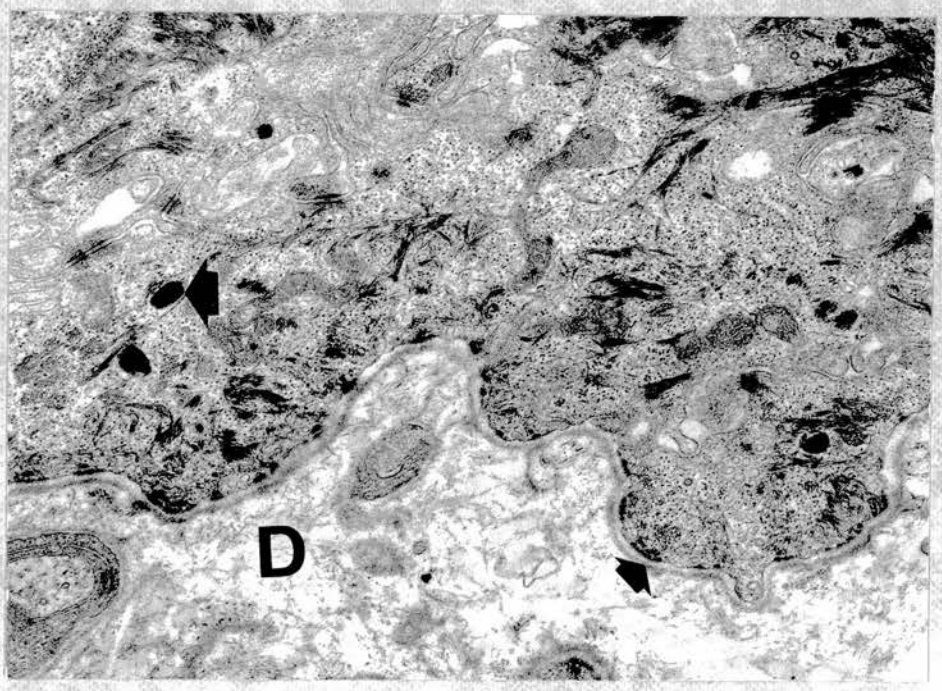


Plate 7 (X 16,000): Basal cells and basal lamina. Note melanosomes dispersed singly (large arrow). Small arrow points to hemidesmosome. D, dermis.

Two types of cell can be distinguished in the horny layer; one has a homogenous matrix and the other looks more fibrillar and spongey. At this level the cell walls appear thicker and variable amounts of granular or reticular material may be seen in the intercellular spaces (Plate 9). The desmosomes also appear different at this level as there is now no dense attachment plaque associated with the inner aspect of the plasma membrane (Plate 9). In tanned skin melanosomes can also be easily seen in cells of the horny layer (Plate 9).

The appearance of the intercellular space is variable. If fixation is good, and no local anaesthetic is used when the biopsy is obtained, then it may be very narrow and even difficult to make out (Plates 3 and 7). If wider, it may contain no obvious contents or an amorphous material. Dendritic cell processes are seen in the intercellular space (Plate 4).

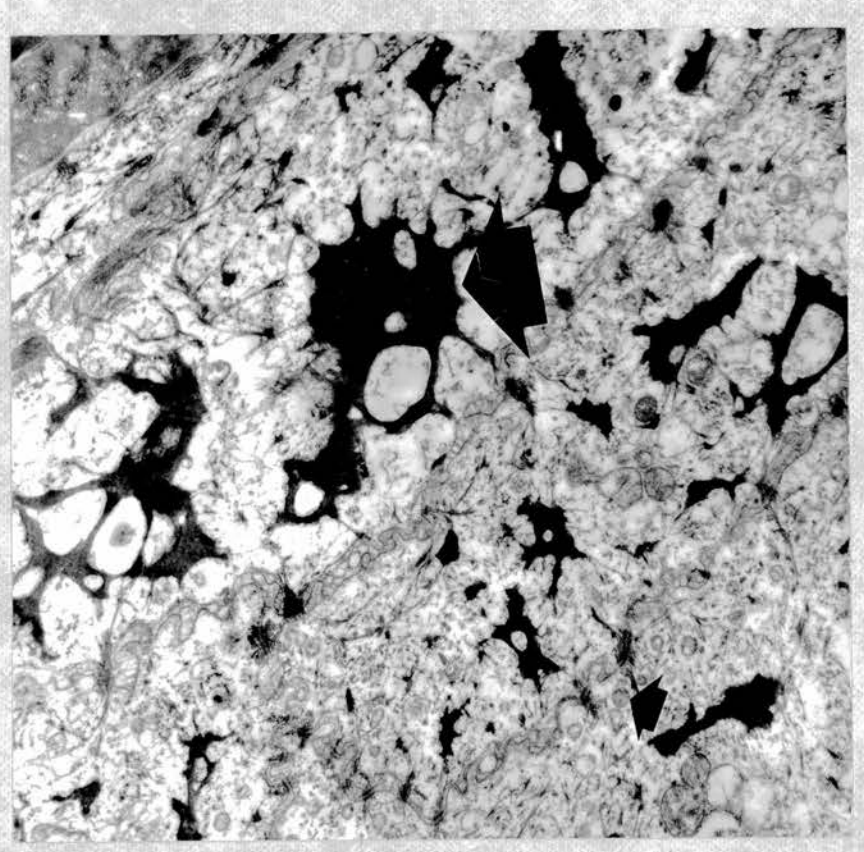


Plate 8 (X 13,000): Granular cell layer
Horny cells top left. Large arrow points to keratohyalin granule. Small arrow indicates membrane coating granule.

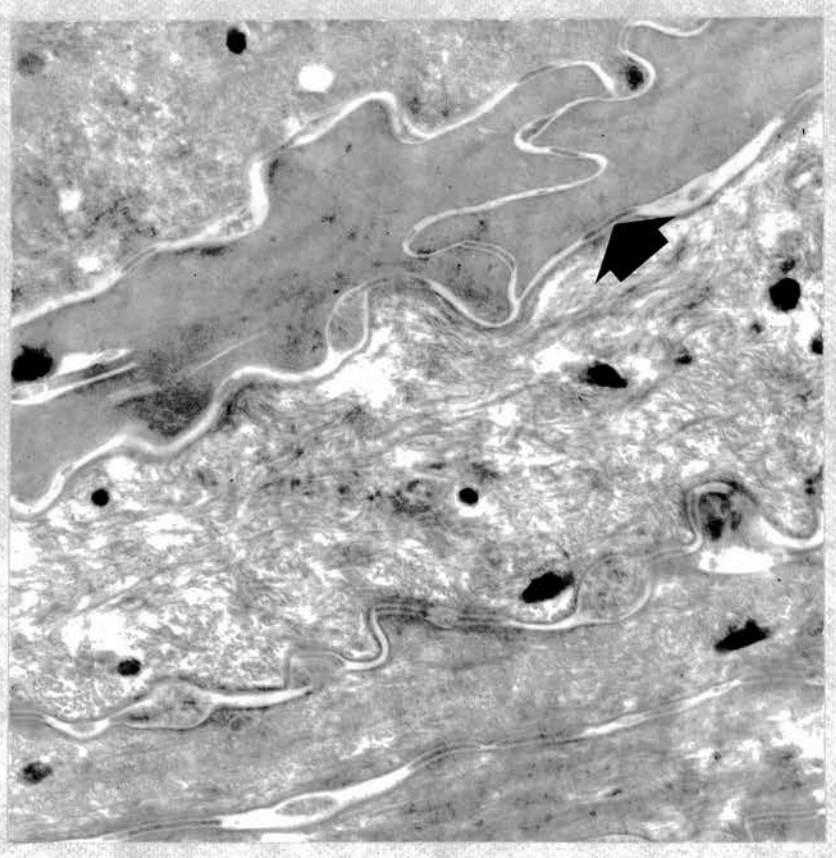


Plate 9 (X 22,000): Horny layer
Note two types of cell and melanosomes in cells.
Arrow indicates remnant of desmosome.

(2) The melanocyte

At the ultrastructural level the melanocyte is a distinctive cell. It is distinguished from neighbouring keratinocytes by its lack of tonofibrils, and desmosomes are absent. It is seen most often in the basal cell layer (Plates 10 and 12) but sometimes seems to suspend from it into the dermis (Plate 13). Unlike the keratinocyte, mitochondria are abundant but, curiously, are often poorly preserved compared with those in other cell types (Plates 10 and 13). Microfibrils, as distinct from tonofilaments, are seen in the cytoplasm. Unlike tonofilaments they show no tendency to form bundles and are often seen as parallel arrays of fine filaments. The Golgi apparatus is usually prominent (Plates 10 and 12) and the endoplasmic reticulum well developed.

The cell has a smooth plasma membrane which shows occasional thickened areas when apposed to the basal lamina (Plate 13). There are however no hemidesmosomal attachments to the basal lamina (Plates 10, 12 and 13).

The characteristic organelle of the melanocyte is the melanosome (Plate 11). These are numerous in melanocytes of negroids and individuals with a dark complexion (Plate 10) though they are seen without difficulty in the melanocytes of those with a fair skin (Plate 12). They are ovoid or rod shaped bodies measuring 0.4 - 1.0μm in length and 0.1 - 0.5μm in diameter. The nomenclature of the four stages has

been described already in Chapter I (page 25). Stage I melanosomes are seen as spherical vesicles near the Golgi apparatus. The other stages are usually seen scattered singly throughout the cytoplasm (Plate 10) though there is a proponderance of Stage III and Stage IV melanosomes in the dendritic processes (Plate 4). If preservation is good a distinct unit membrane can be seen surrounding the internal structure of the organelle (Plate 11). Occasionally complexed melanosomes (as in Plates 5 and 6) are also seen in normal melanocytes.

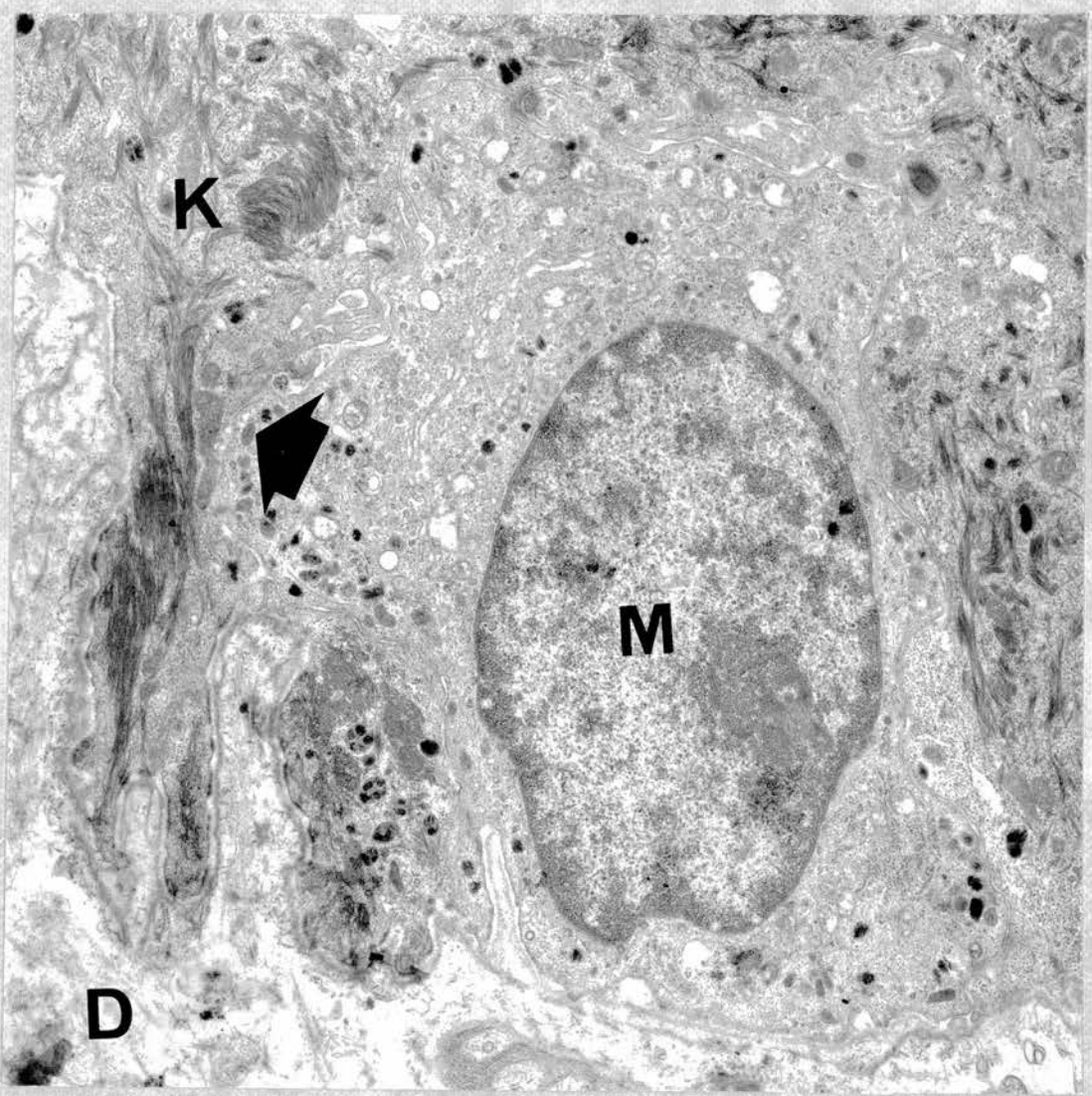


Plate 10 (X 13,500): Melanocyte (M) on basal lamina (Caucasoid with dark complexion). Arrow indicates melanosome. K, keratinocyte; D, dermis.

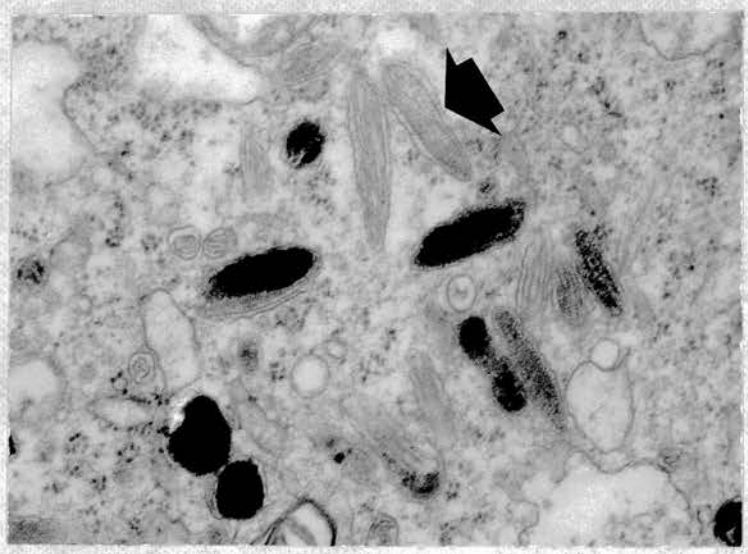


Plate 11 (X 38,000): Melanosomes of Stages II, III and IV in melanocyte.
Note filaments in some with cross connections, causing striation with periodicity of 80 - 100A° (arrow).

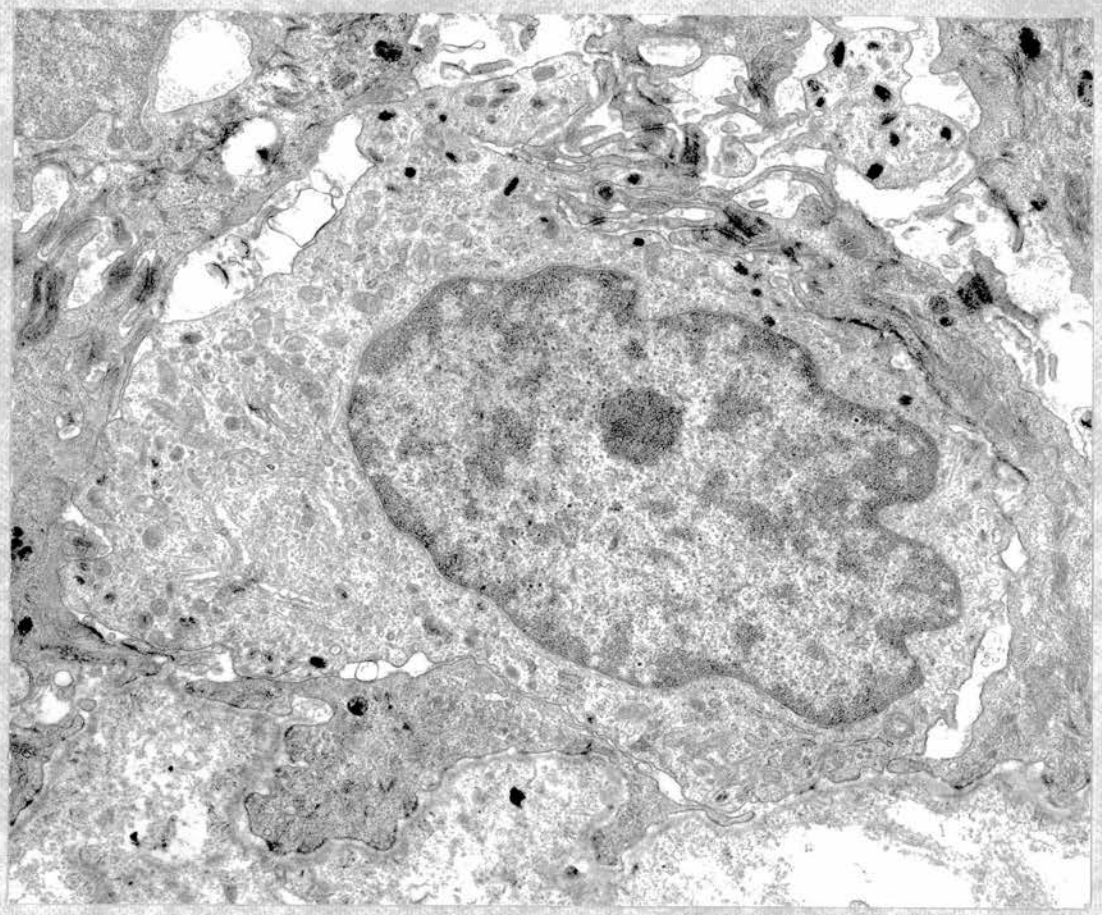


Plate 12 (X 15,500): Melanocyte on basal lamina (Caucasoid with fair complexion). Note relatively few melanosomes.

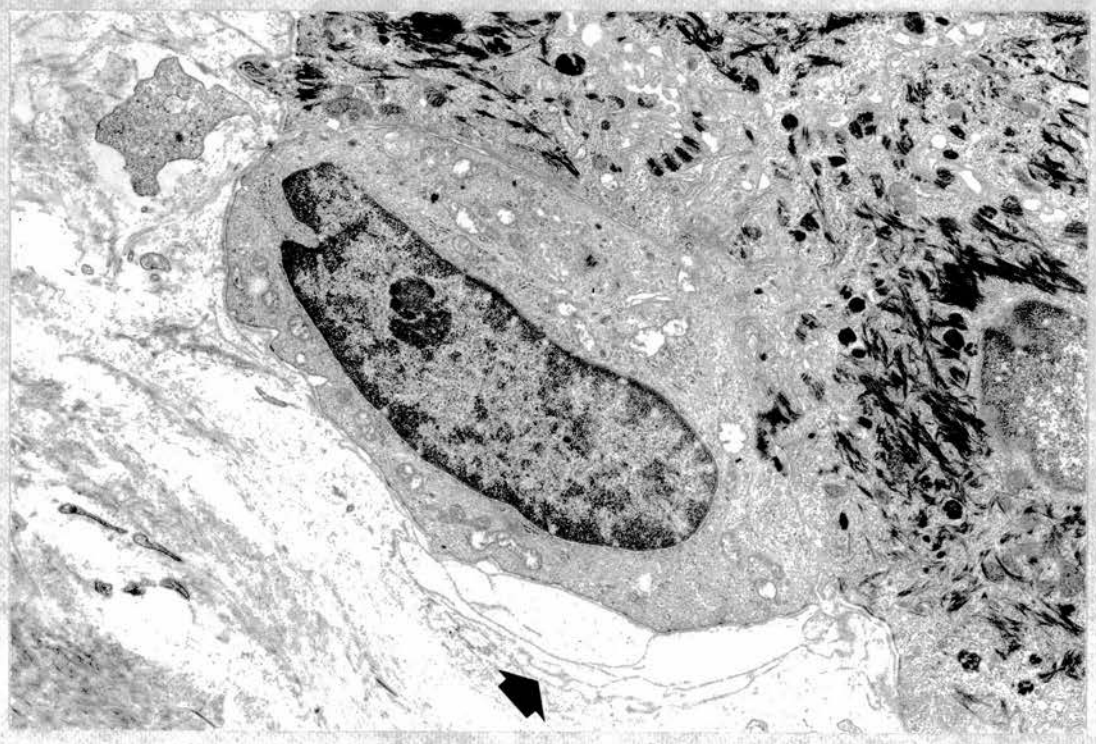


Plate 13 (X 8,000): Melanocyte "suspended" from basal layer. Note replicated basal lamina in this normal skin (arrow).

(3) The Langerhans cell

Like the melanocyte the Langerhans cell is clearly separable from the surrounding keratinocytes (Plate 14). It is usually seen in the mid epidermis, but in normal skin may be near, or on, the basal lamina (Plate 15). Its cytoplasm appears clear as it does not contain tonofibrils and the nucleus is often convoluted. The perinuclear space is also more prominent than in keratinocytes. The plasma membrane lacks the villi seen in the keratinocyte but is more ruffled than that of the melanocyte. The cytoplasm contains numerous mitochondria and a particularly prominent Golgi apparatus. Occasional cytoplasmic microfilaments are present and lysosomes with a granular or lamellar matrix are frequent (Plate 16). Centrioles are seen frequently (Plates 15 and 16).

The organelle which distinguishes the Langerhans cell from others is the Langerhans cell granule (see Chapter I page 39). In routine sections these are most commonly seen as rod or racket shaped profiles (Plates 16 - 20). However more careful observation will reveal other profiles which are due to oblique cuts through granules (Plates 16 - 20). These can only be explained if the granule is considered as a flat plate-like structure with a spherical or hemispherical blob protruding from its surface (see Chapter I page 41). The granules are scattered throughout the cytoplasm

and, in normal skin, are rarely seen attached to the cell wall (Plate 20).

The vesicular portion of the granule has a clear centre, and the internal face of its membrane has a fuzzy coat which, in some sections, can be seen to consist of regularly spaced granular particles. These particles are continuous with those lining the outer membrane of the rod-like portion (Plate 16).

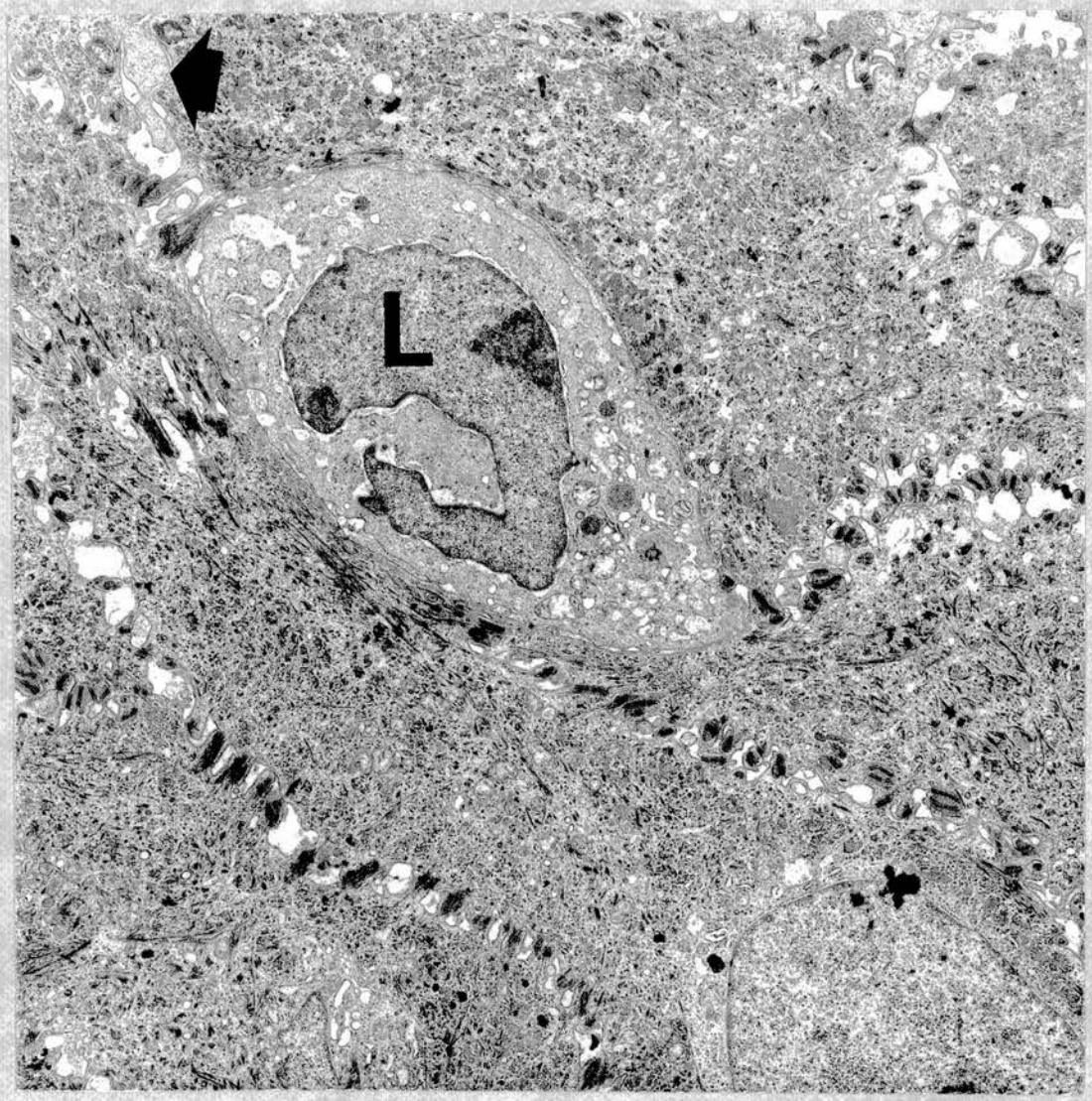


Plate 14 (X 6,000): Epidermal Langerhans cell (L)
Note convoluted nucleus, lack of tonofibrils in cells (c.f. neighbouring keratinocytes) and no desmosome connections with surrounding cells. Langerhans cell granules are just visible. Arrow indicates dendritic process of Langerhans cell.

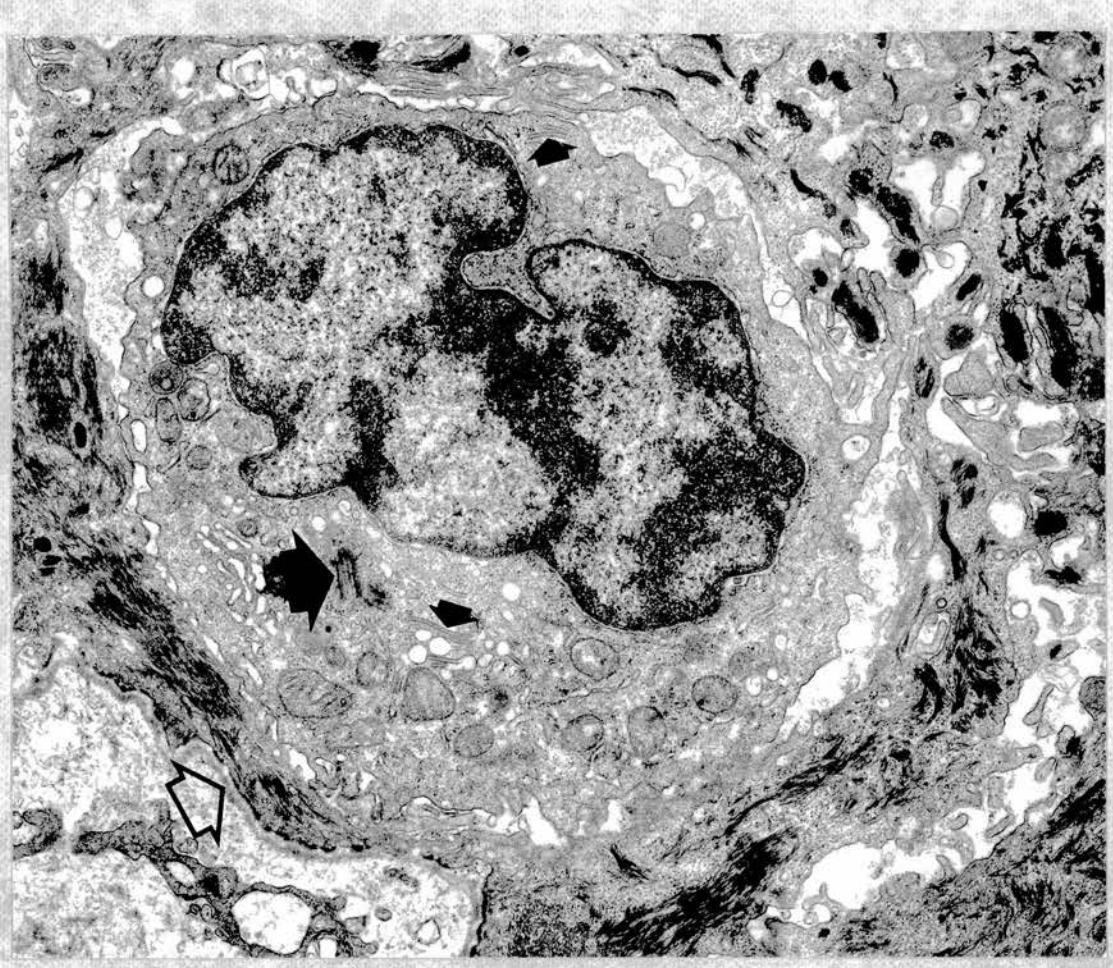


Plate 15 (X 15,000): Langerhans cell near basal lamina (open arrow). Large arrow points to centriole. Small arrows indicate granules.

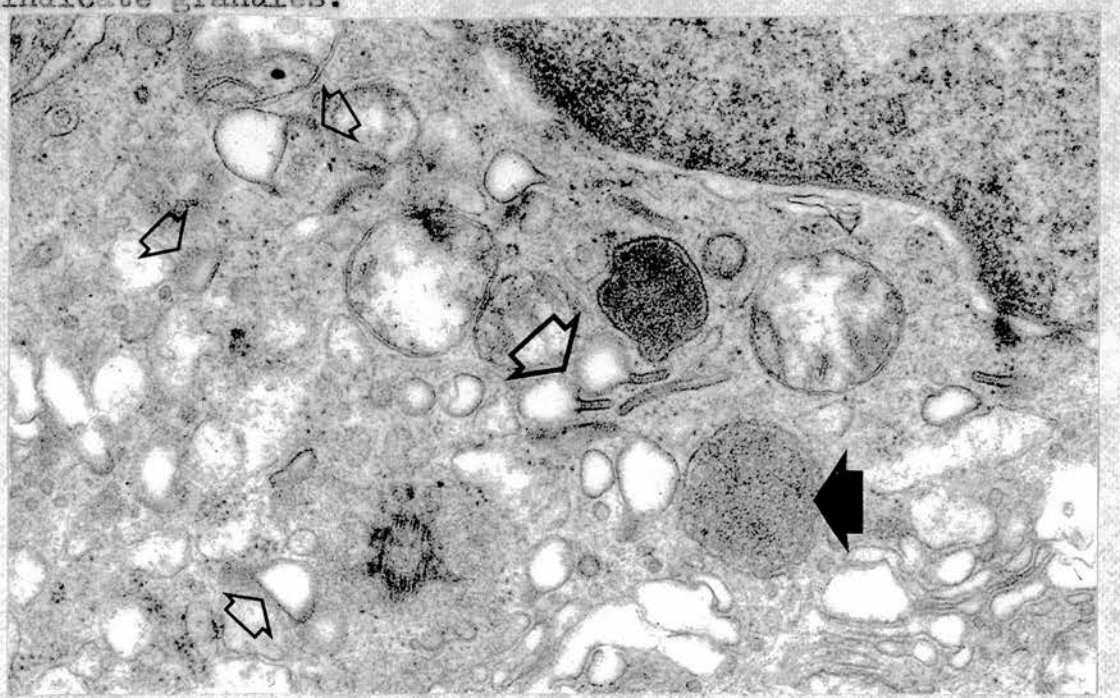


Plate 16 (X 36,000): Golgi region (bottom right) of Langerhans cell. Nucleus top right. Black arrow indicates granular lysosome. Large open arrow points to "tennis racket" profiles of granules. Small open arrows indicate other granule profiles (c.f. Fig. 9).

Plate 17 (x 29,000):
Langerhans cell granules (black arrows) showing periodicity of central lamella.

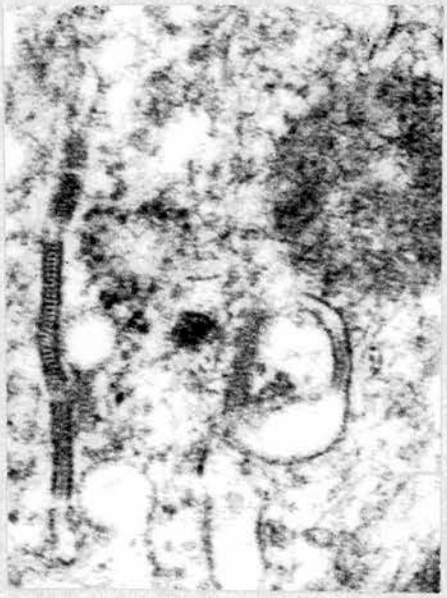


Plate 18 (X 67,000):
Langerhans cell granules showing striated appearance (90A° periodicity)

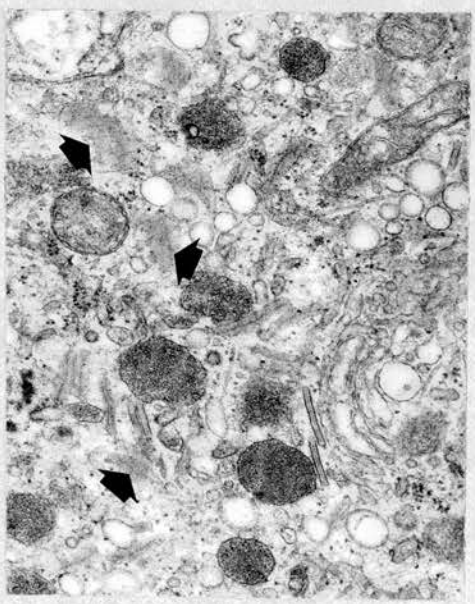


Plate 19 (X 21,000):
Langerhans cell granules (arrows) showing crystalline structure.

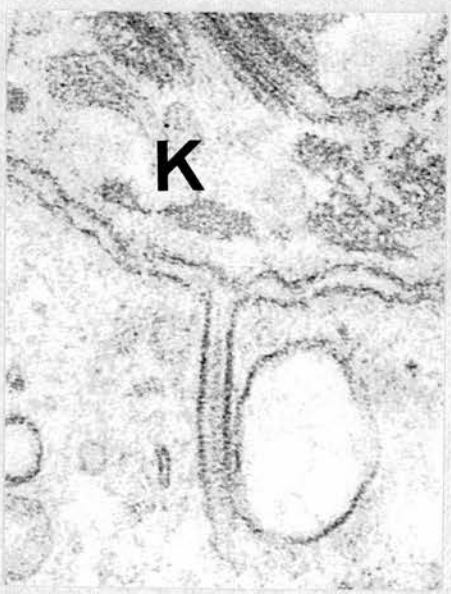


Plate 20 (X 108,000):
Granule attached to Langerhans cell wall
K, keratinocyte

4. DISCUSSION

The fine structure of the dendritic cells noted here is similar to that reported in standard reviews of them (e.g. Zelickson, 1967; Breathnach, 1971 and Wolff, 1972). However the observation that mitochondria of normal melanocytes seem particularly prone to distension and rupture does not seem to have been recorded elsewhere, though communication with other workers, and examination of published micrographs, confirms that it is not uncommon. Its recognition is important only in that such changes cannot be attributed to any pathological event in other situations.

Chapter IV

SUBCELLULAR RESPONSES IN DENDRITIC CELLS TO ULTRAVIOLET IRRADIATION.

Including

1. INTRODUCTION

2. MATERIAL AND METHODS

3. RESULTS

4. DISCUSSION
   a) Subcellular responses
   b) Cellular responses

1. INTRODUCTION

Until 1968 most knowledge on the subcellular localisation of melanin biosynthesis had been gained by the study of malignant melanoma, using techniques involving ultra-centrifugal separation of cell particles and density gradient centrifugation. The separated fractions were then subjected to both enzymatic analysis and electron microscopic examination, and inferences were made regarding the role of various organelles in melanogenesis (see Chapter I and Fitzpatrick et al., 1967). In 1968 Novikoff et al. described ultrastructural and cytochemical observations on the origin of melanosomes, again studying neoplastic melanocytes in mice. The following studies were therefore undertaken to determine the subcellular sites of melanogenesis in normal human melanocytes.

As the work of Novikoff et al. (1968) had highlighted the role of the Golgi apparatus in melanogenesis, particular attention was paid to this region (shown schematically in Fig. 11). The term saccule is preferred to cisterna, and the two faces of the apparatus are called "vesicular" and "vacuolar" rather than "forming" and "mature" in order to avoid preconceived notions on their function (Mollenhauer and Whaley, 1963). The system of smooth endoplasmic reticulum closely associated with the Golgi apparatus is referred to as GERL according to the terminology of

Novikoff (1967) and Novikoff et al. (1968). The initials indicate that it is part of the endoplasmic reticulum (ER), closely related to the Golgi apparatus (G) and produces lysosomes (L). Serial sectioning has revealed GERL to be continuous with the rough endoplasmic reticulum (Novikoff et al., 1966) and the Golgi apparatus (Maul, 1969).

2. MATERIAL AND METHODS

Melanogenesis was induced by a single exposure of ultraviolet radiation. Its subcellular localisation was demonstrated by detecting tyrosinase activity, using the ultrastructural dopa reaction described by Mishima (1962), and the light microscopic tyrosine reaction of Fitzpatrick et al. (1950) adapted for electron microscopy.

The untanned skin of the upper forearm of a 30 year old caucasoid male was selected as the test site. The minimal erythema dose (MED) for this area using a hot quartz lamp source (Burdick Company, Milton, Wisconsin. Type QA 450. Peak transmission at the 309 nm and 313 nm bands) was established. An area of approximately 2 cm^2 was then irradiated with a dose equivalent to 6 X MED. This caused visible erythema in 1 - 2 hours following irradiation and slight clinical oedema by 24 hours, when the reaction appeared to be at its height. Vesiculation was never noted and

the treated area always showed residual pigmentation when the erythema had disappeared (approx. 120 hours following irradiation). A 3mm punch biopsy was taken from the test area after irradiation and at the following intervals: 2 hours, 6 hours, 24 hours, 72 hours and 120 hours. Ultrastructural dopa, tyrosine and control reactions were carried out on each biopsy specimen.

Ultrastructural dopa reaction

The tissue was fixed in cold 2.5% glutaraldehyde 0.1M phosphate buffer at pH 6.8 and sliced into thin sections of approximately 100μm thick after 90 minutes. Total fixation time was 2 - 3 hours. The sections were then thoroughly rinsed in 0.1M phosphate buffer at pH 6.8 and transferred to the incubating medium containing 0.1% L-3, 4-dopa freshly prepared in 0.1M phosphate buffer at pH 6.8. The slices were kept in this overnight at 3 - 4°C and then transferred to fresh incubating medium at 37°C. After 7 hours incubation the sections were rinsed and post-fixed in cold buffered 1% osmium tetroxide for 1 hour.

Ultrastructural tyrosine reaction

The tissue was again fixed in cold 2.5% glutaraldehyde 0.1M phosphate buffer at pH 6.8 and sliced into thin sections of approximately 100μm after 90 minutes. Total

fixation time was 2 - 3 hours. Following rinsing in 0.1M phosphate buffer at pH 6.8, the sections were transferred to the incubation medium containing 0.05% L-tyrosine freshly prepared in 0.1M phosphate buffer at pH 6.8. After incubation overnight at 3 - 4°C the sections were transferred to fresh incubation medium and incubated at 37°C for 24 hours. The sections were then rinsed and postfixed in cold osmium tetroxide.

Control reaction

This was carried out exactly as for the dopa and tyrosine reactions except that the incubation medium did not contain any substrate in the 0.1M phosphate buffer at pH 6.8.

Other methodological details are as outlined in Chapter II.

3. RESULTS

Following ultraviolet irradiation the dopa and tyrosine reaction product is specifically deposited within the melanocytes of the epidermis (Plate 21). Keratinocytes and Langerhans cells do not contain reaction product (Plates 21 and 23), while non-irradiated melanocytes, incubated in dopa or tyrosine, may contain vesicular but not linear reaction product (see below).

The cellular and subcellular localization and nature of the reaction product using both substances as substrates are essentially identical. With the methods used, reaction product is more commonly seen when dopa is the substrate, though subcellular morphology has not been so well preserved as when tyrosine is used. For this reason, the micrographs are mainly from sections incubated in tyrosine.

Linear reaction product

Linear reaction product has been seen in:

1) smooth endoplasmic reticulum not clearly associated with the Golgi apparatus;
2) smooth endoplasmic reticulum closely associated with the Golgi apparatus (GERL), this being the most common site (Plates 22, 24, 25 and 27);
3) Golgi saccules (Plates 24 and 28).

The linear reaction product often appears to have broken down into small vesicles containing reaction product (Plates 24, 25, 27 and 28) and frequently similar vesicles bud from the ends or sides of saccules containing reaction product (Plates 24 and 28).

Vesicular reaction product

Vesicular reaction product has been seen in:

1) Golgi vesicles and vesicles closely associated with Golgi apparatus (GERL) (Plates 22 and 24 - 28); it is often impossible to differentiate between these;
2) intracytoplasmic vesicles of all sizes ranging from 50nm to 500nm in diameter (Plates 22 and 24 - 28).

At times, melanocytes demonstrating reaction product contain Stage II, III and IV melanosomes in which there is no deposition of the reaction product (Plate 26).

Rarely, a diffuse darkening of the cytoplasm of dopa-incubated melanocytes was noted. This seemed to be attributable to section thickness, but also tended to occur at times when numerous vesicles containing reaction product were present (e.g. 120 hours after irradiation).

Plate 21 (X 10,000): Melanocyte with reaction product 72 hours following ultraviolet irradiation.
Incubation:tyrosine.
Note reaction product (arrows) in melanocyte, but not in surrounding keratinocytes.

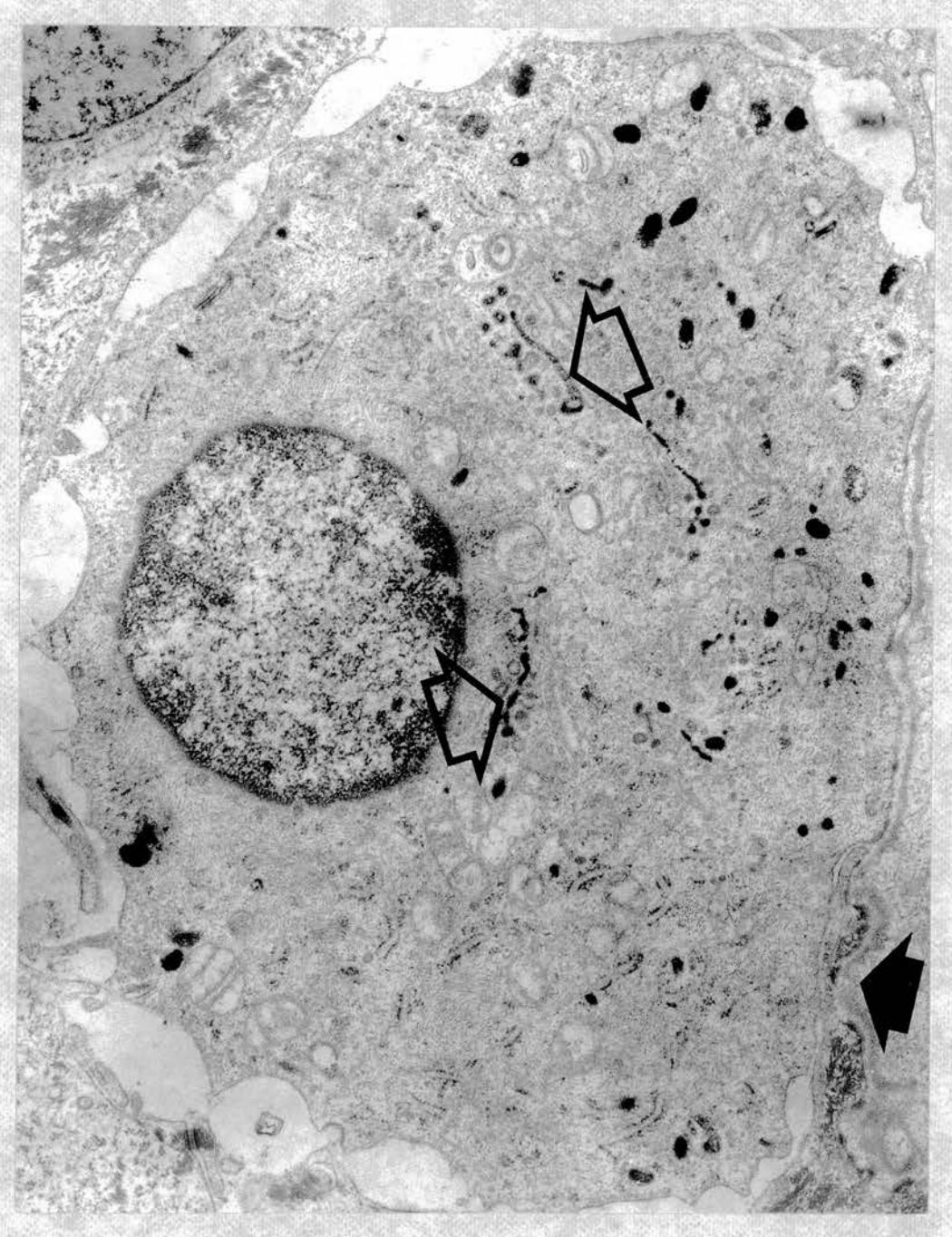


Plate 22 (X 18,000): Melanocyte with reaction product 72 hours following ultraviolet irradiation. Incubation:tyrosine. Melanocyte on the basal lamina (arrow). Linear reaction product (open arrows) together with vesicles containing reaction product are seen in close association with the Golgi apparatus. Numerous vesicles of varying size, and containing reaction product are seen between those associated with the Golgi complex and the melanosomes at the cell periphery.

Plate 23 (X 13,000): Langerhans cell with no reaction product 72 hrs. following ultraviolet irradiation. Incubation:tyrosine.

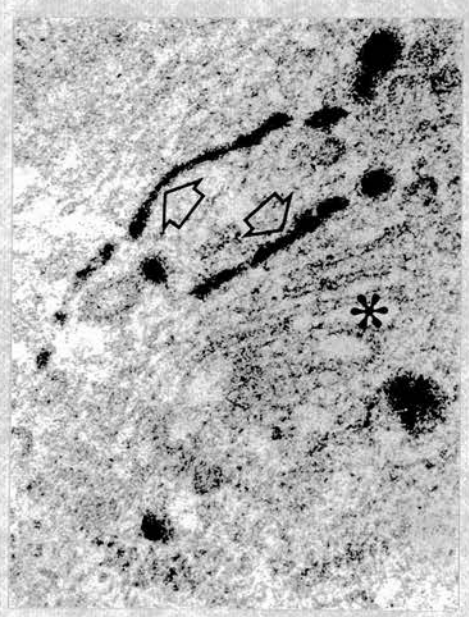


Plate 24 (X 59,000):
Reaction product
72 hrs following ultraviolet irradiation. Incubation: tyrosine. Golgi region of a melanocyte. Arrows point to linear reaction product in GERL (upper left), and in the peripheral saccule at the vesicular face of the Golgi apparatus (lower right). They are separated by vesicles containing reaction product.
* - Golgi saccule.

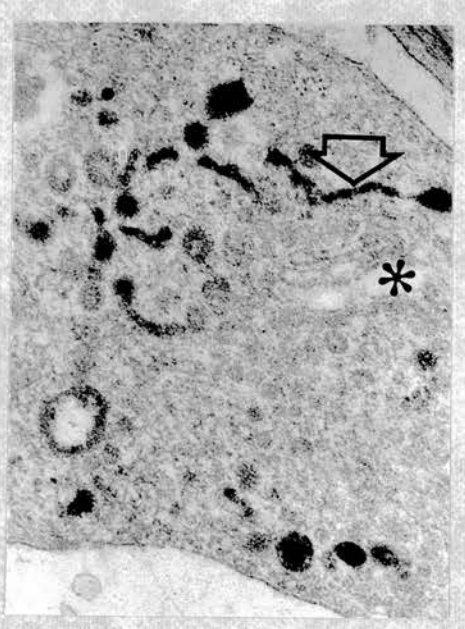


Plate 25 (X 40,000):
Reaction product
72 hrs following ultraviolet irradiation. Incubation: tyrosine. Golgi region of a melanocyte. Arrow points to reaction product in GERL. Note numerous vesicles containing reaction product, some of which appear to be budding from the linear reaction product.
* - Golgi saccule.

Plate 26 (X 50,000):
Reaction product
72 hrs following ultraviolet irradiation. Incubation:dopa. Note that, in spite of linear and vesicular reaction product (arrows), there are areas showing the typical matrix pattern of melanosomes containing no reaction product (open arrows).

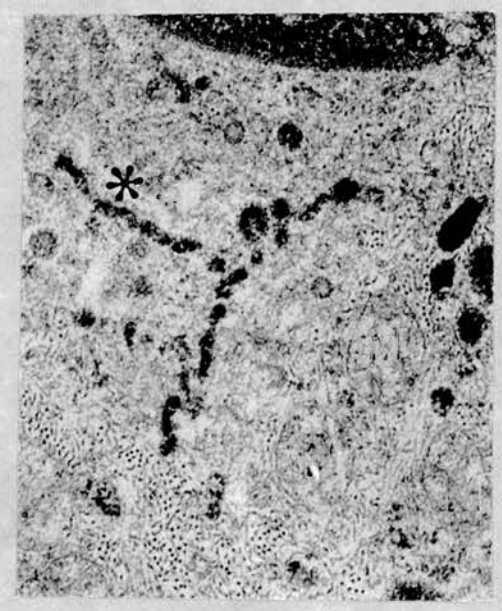


Plate 27 (X 50,000)
Reaction product
72 hrs following ultraviolet irradiation. Incubation:tyrosine. Golgi region of a melanocyte. The linear reaction product appears as numerous small vesicles.
* - Golgi saccule.

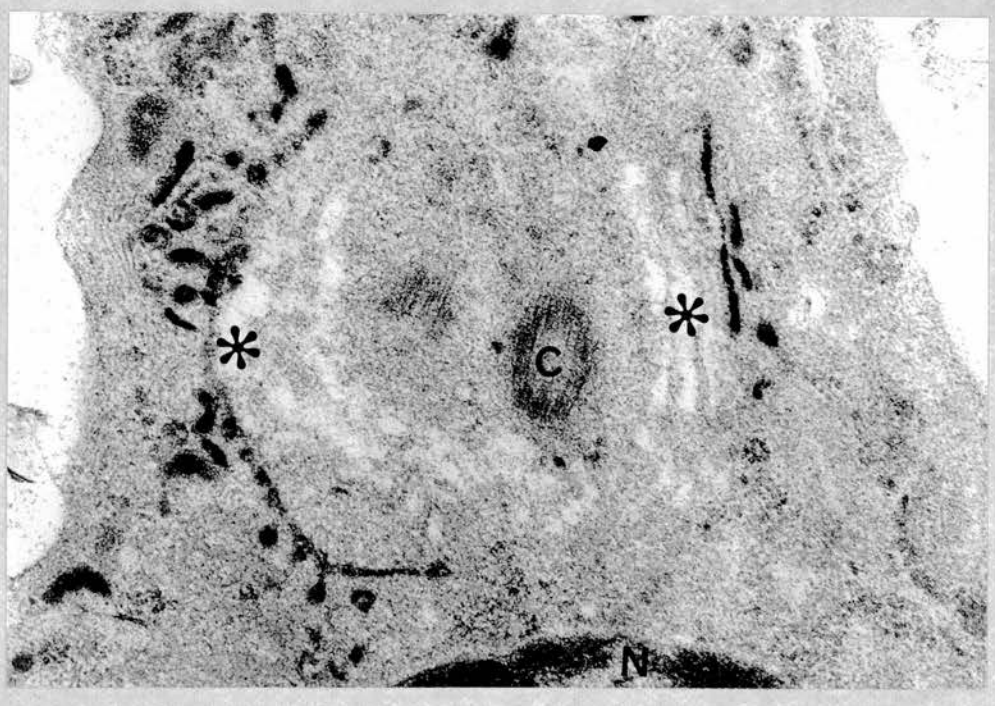


Plate 28 (X 46,000): Reaction product
72 hrs following ultraviolet irradiation. Incubation:dopa. Golgi region of a melanocyte. Reaction product is seen in the peripheral saccules at the vesicular face of the Golgi complex, with stacking on the right. Note small vesicles containing reaction product budding from some places.
N - Nucleus; C - Centriole; * - Golgi saccule.

The subcellular localization of the reaction product (summarized in Table 1) is dependent on the interval following irradiation. Linear reaction product was not seen until 24 hours following irradiation. At this time a higher proportion of the reaction product was present in a linear form in the smooth endoplasmic reticulum associated with the Golgi apparatus, while at 120 hours post irradiation more was seen in discrete vesicles of all sizes.

Cellular and subcellular localization of reaction product

Location	Incubated in DOPA or tyrosine						Incubated in buffer		
	Non-irrad.	2 hr.	6 hr.	24 hr.	72 hr.	120 hr.	Non-irrad.	24 hr.	72 hr.
Epidermis									
Melanocyte									
Cytoplasm (darkening)	–	–	–	–	–	+	–	–	–
Mitochondrion	–	–	–	–	–	–	–	–	–
Smooth endoplasmic reticulum	–	–	–	+	+	–	–	–	–
Golgi region									
a) Associated smooth endoplasmic reticulum (GERL)	–	–	–	++	+++	+	–	–	–
b) Peripheral saccule and vesicles at the vesicular face	–	–	–	+	+++	++	–	–	–
c) Saccules	–	–	–	+	+	–	–	–	–
d) Peripheral saccule and vacuoles at the vacuolar face	–	–	–	–	+	+	–	–	–
Intracytoplasmic vesicles (100–500 mμ)	+	+	+	+	+++	+++	–	–	–
Langerhans cell	–	–	–	–	–	–	–	–	–
Keratinocyte	–	–	–	–	–	–	–	–	–
Dermis									
Mast cell	–	–	–	–	–	–	–	–	–
Melanophage	–	None seen	–	–	–	None seen	–	–	–

– No reaction product noted, + Reaction product rarely noted, ++ Reaction product commonly noted, +++ Reaction product often noted.

Table 1: Localization of reaction product

Reaction product was not noted in mast cells or melanophages. Grids with numerous melanocytes containing reaction product often had mast cells with characteristic "finger printing" of their granules (indicating no reaction product within) and no reaction product in other areas.

4. DISCUSSION

a) Subcellular responses to ultraviolet irradiation

The ultrastructural cytochemical procedures used in these experiments proved relatively simple and the results were readily reproducible. Presumably this was in part due to the reaction product (melanin) being electron dense and not requiring the addition of a "capturing" reagent for its demonstration (Hunter, 1972a).

The distribution of reaction product in both the dopa and tyrosine experiments is very suggestive of an important role for the Golgi region in the process of melanogenesis. Fig. 11 is a composite diagram of the Golgi region in a melanocyte. The subcellular localisation of reaction product noted in the above experiments is illustrated on the right of the interrupted line.

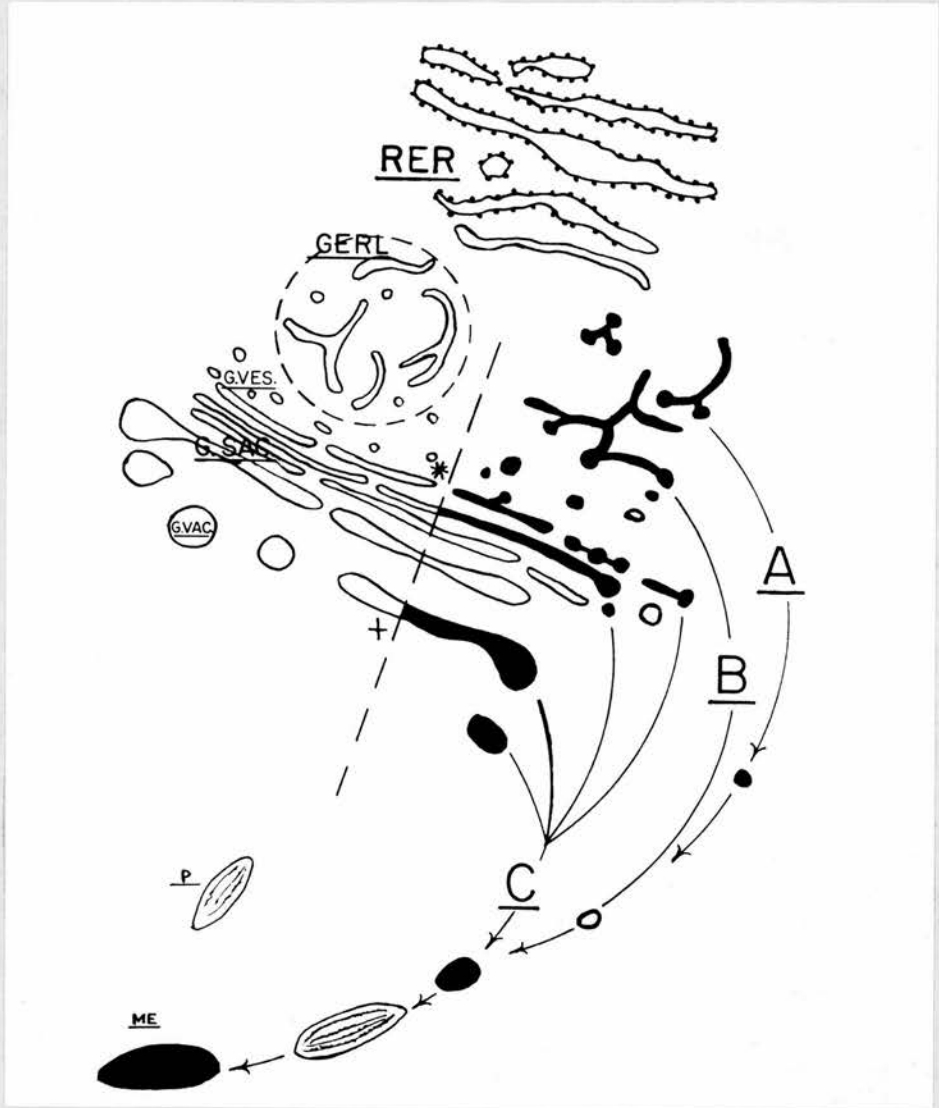


Figure 11: Composite diagram of the Golgi region in a melanocyte To the left of the interrupted line the Golgi apparatus has been labelled according to the terminology used in this Chapter. G.VES, Golgi vesicles; G.SAC, Golgi saccules; G.VAC, Golgi vacuoles; * Vesicular face of the apparatus; + Vacuolar face of the apparatus; GERL, Golgi associated system of smooth endoplasmic reticulum; RER, Rough endoplasmic reticulum. The subcellular localization of reaction product is illustrated at the right of the interrupted line. The arrows indicate suggested pathways in the development of the melanosome (ME). A. From smooth endoplasmic reticulum. B. From smooth endoplasmic reticulum associated with the Golgi complex (GERL). C. From all parts of the Golgi apparatus. Of these, B pathway appears to be the most common. Note the structures resembling the matrix of melanosomes (P) which contain no reaction product, even though it is present in surrounding vesicles.

Biochemical analysis, autoradiography and ultrastructural morphology have indicated that secretory proteins are concentrated and packaged at various sites within the Golgi region, depending on the cell type examined. This occurs, for example, in condensing vacuoles in guinea pig pancreas (Caro and Palade, 1964; Jamieson and Palade, 1967), in vesicles derived from opposite faces of the Golgi apparatus in rabbit polymorphonuclear leukocytes (Bainton and Farquhar, 1966), in vacuoles derived from Golgi saccules in rat anterior pituitary cells (Smith and Farquhar, 1966) and in a Golgi-associated system of smooth endoplasmic reticulum in cells of rat adrenal medulla and mouse melanoma (Holtzman and Dominitz, 1968; Novikoff et al., 1968). Our observations support the concept that tyrosinase may be packed in all of these regions in the human melanocyte to a lesser or greater degree.

Novikoff (1967), on the basis of a high acid phosphatase content and its relationship with coated vesicles and lysosomes, considers this Golgi-associated system of smooth endoplasmic reticulum to have a special functional significance. The above experiments would certainly confirm its prime importance in the process of melanogenesis. The observations suggest that most vesicles containing tyrosinase make their earliest appearance from this area, and vesicles of gradually increasing size are seen between here and the cell

periphery. The production of such vesicles would not appear, in this instance, to involve the Golgi apparatus at all. It is difficult to define the mechanism whereby developing melanosomes increase in size as vesicles containing reaction product have not been noted to fuse together.

Tyrosinase activity has also been detected within Golgi saccules, more often at the vesicular face (Plates 24 and 28) than at the vacuolar face. Vesicles containing tyrosinase appear to bud from these regions as well (Plate 28), indicating other pathways of melanosomal production.

Linear reaction product could not be demonstrated in the melanocytes which were not exposed to ultraviolet irradiation. The tyrosinase levels in these areas are apparently too low for detection by this technique. Ultraviolet irradiation causes a burst of tyrosinase activity of limited duration, and the newly synthesized enzyme can be detected as indicated in Table 2.

The findings of the experiments described above have been accepted by other workers (Lever, 1975 and Rupec, 1973) and the method is now used routinely for the ultrastructural demonstration of active melanin synthesis within a cell (e.g. Konrad et al., 1974).

Toda and Fitzpatrick (1971) have warned that it would be unwise to infer that there is no tyrosinase activity in Stages II, III and IV melanosomes, when

glutaraldehyde prefixation is used (as in the above experiments). They have shown that such prefixation inactivates tyrosinase in isolated melanosomal fractions prepared from embryonic chick retinal pigment epithelium. In their ultrastructural dopa studies they noted reaction product in the surrounding membrane of Stage II, III and IV melanosomes when the tissue was incubated in dopa without prefixation. However their findings were otherwise similar to those above regarding the localisation of tyrosinase in the Golgi and surrounding region. They summarised them:

> "Tyrosinase is synthesized in the ribosome; it then transfers to the Golgi area and attaches to the membrane; the membrane balloons out from the smooth-surface endoplasmic reticulum in the Golgi area, and the Stage I melanosome is formed, in which melanization begins. In the Stage II melanosome, the fine structure with periodicity is not the supporting structure of tyrosinase."

Fig. 12, taken from Toda and Fitzpatrick's paper depicts these views.

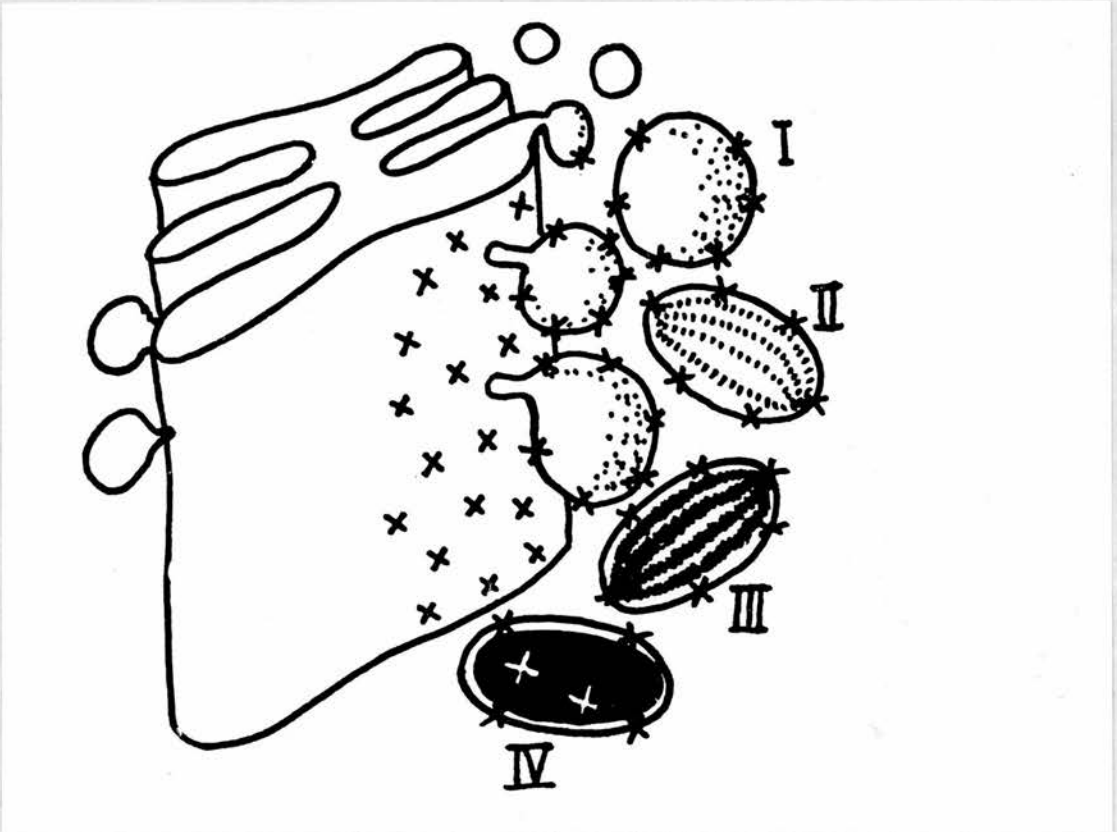


Figure 12: The formation of the melanosome (X = tyrosinase) (From Toda and Fitzpatrick, 1971)

Maul's (1969) studies on the Golgi-melanosome relationship in human melanoma cells in vitro are also worth mentioning. His findings are not so different from those reported above as first appears. By means of three-dimensional reconstruction of serial sections, he concluded that the earliest melanosomal structure (with periodicity) developed within focal dilatations of the tubular smooth endoplasmic reticulum (i.e. GERL) which is connected with the Golgi during melanogenesis. He noted that the frequency with which connections between melanosomes and smooth

endoplasmic reticulum could be observed decreased with progressive melanization. He went on to suggest that tyrosinase might be channelled from the Golgi complex through tubular membrane structures into early melanosomes, because there seemed to be tubular connections between these and elements of the smooth endoplasmic reticulum as well as the Golgi (Fig. 13). Such an idea would fit in very well with our experimental results.

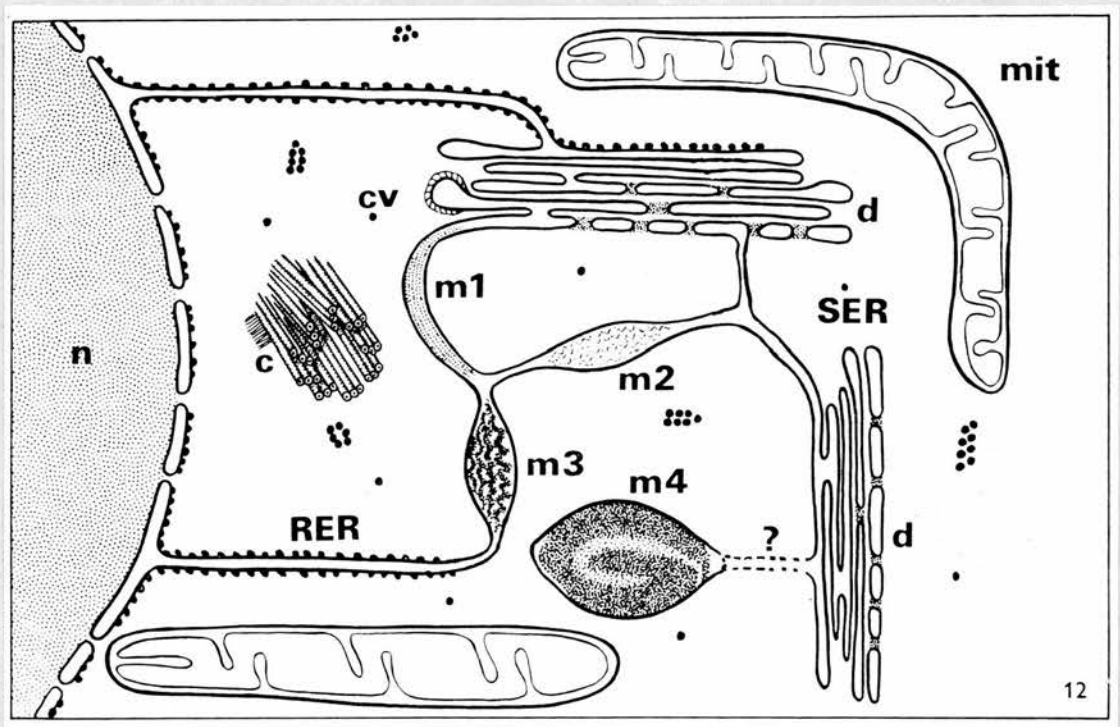


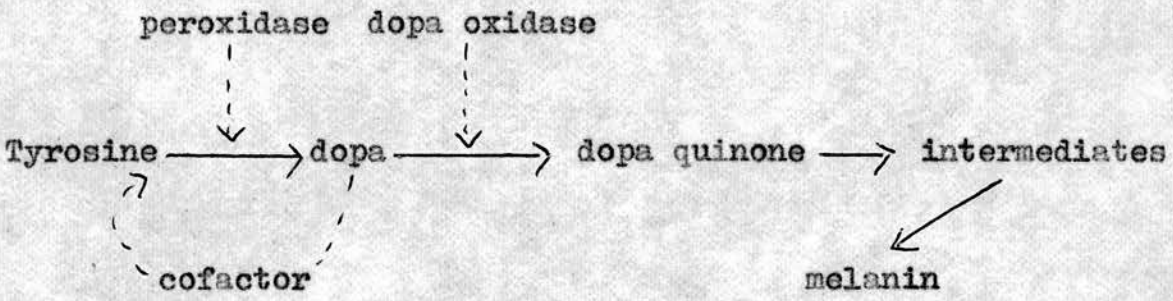
Figure 13: Maul's scheme for melanosomal formation

A schematic drawing of the relationship of the Golgi apparatus (d), coated vesicles (cv), tubular smooth endoplasmic reticulum (SER), melanosomes (m1 - m4), rough endoplasmic reticulum (RER) and centriole (c).
M1 - M4 melanosomes are in successive stages of development:
M1 slightly dilated SER with longitudinal fibres forming
M2 helix formation and shortening of the matrix fibres
M3 beginning of melanin deposition
M4 melanosome close to completing melanin deposition; the connection with SER is resolved.

In another study, using an ultrastructural dopa reaction on regenerating fowl feather, Maul and Brumbaugh (1971) proposed a rather different mechanism by which tyrosinase becomes associated with the earliest melanosome. They found reaction product in a situation identical to that noted in our experiments. As they were unable to demonstrate tyrosinase activity in early melanosomes they assumed that vesicles containing the enzyme (seen near the Golgi) fused with early melanosomes, though no evidence for this was seen. However Toda and Fitzpatrick's (1971) findings of melanosomal tyrosinase inhibition in specimens prefixed with glutaraldehyde make such an explanation unnecessary.

After the experiments described in this Chapter were completed, Okun and his associates questioned the validity of assuming that the reaction product, produced in the ultrastructural tyrosine and dopa reaction, is formed by tyrosinase action. A series of experiments (see Okun et al., 1973a) have led his team to suggest that mammalian melanogenesis may be based on the synergistic action of two enzymes, an aerobic dopa oxidase and a peroxidase. They consider that the oxidation of tyrosine to melanin by peroxidase is peroxide dependent and that hydrogen peroxide is generated by various oxidases and by auto-oxidation of intermediates in melanogenesis. They feel that the traditional view of tyrosinase activity is incorrect

(see Chapter I) and should be modified:



The conclusions from experiments such as those described in this Chapter and others (Fitzpatrick et al., 1950) have been criticised on the grounds that peroxidase and catalase (eliminating H_2O_2 availability as substrate for peroxidase) controls were not carried out and prolonged incubation periods (with increased likelihood of non-specific auto-oxidation) were employed.

However non-specific auto-oxidation or oxidation mediated by peroxidase seems unlikely for the following reasons.

1) Lerner et al. (1949) emphasized that at a pH above 7.0 dopa is oxidized in the presence of oxygen without the need for a catalyst, and therefore suggested that an incubation medium with a pH of 6.8 (as used in our experiments) would minimise such auto-oxidation.
2) The characteristic and reproducible pattern of the reaction product does not favour a non-specific random auto-oxidation process.
3) Reaction product was seen only in melanocytes. If

a non specific auto-oxidation of dopa/tyrosine had occurred it is likely that reaction product would have been seen in other cells (e.g. Langerhans cells).

4) Reaction product has been noted by us and others (e.g. Brumbaugh and Zieg, 1972) after shorter incubation periods (e.g. 30 minutes to 3 hours).

5) Peroxidase activity in melanocytes is said to show latency; i.e. some degree of cell damage must be present for its histochemical demonstration (Okun et al., 1973a). Significant cell damage was not produced in this study and the reaction product was seen consistently in well preserved melanocytes.

Okun et al. (1973b), using a similar ultrastructural dopa reaction, agreed that the reaction product noted in GERL is the result of dopa oxidase, rather than peroxidase, activity. Peroxidase activity could not be demonstrated in GERL using the benzidine/diaminobenzidine reaction, and reaction product still occurred in GERL when catalase was present in the medium. However in their experiments on mouse melanoma cells they demonstrated peroxidatic oxidation of tyrosine using dihydroxyfumarate (DHF) as a cofactor. When catalase was added to the tyrosine-DHF-H_2O_2 incubation mixture, formation of reaction product was abolished.

Holstein et al., (1973), however, find little

support for the views of Okun and his colleagues. Multiple forms of tyrosinase were isolated from the pigmented hair bulbs and Harding Passey melanomas of mice using electrophoretic means. These tyrosinases were shown to utilise tyrosine and dopa and to catalyse their oxidation to higher melanogenic intermediates, in the presence of catalase which was sufficient to block peroxidase activity.

Brumbaugh et al., (1973) studied melanogenesis in the fowl using ultrastructural tyrosine and dopa reactions with peroxidase and catalase controls and found no evidence to suggest peroxidatic oxidation of tyrosine or dopa. Their work also indicated that dopa oxidase is capable of using diaminobenzidine as a substrate to form a reaction product! This was confirmed, in vitro, by the production of a diaminobenzidine reaction product by mushroom tyrosinase. Finally they pointed out that the mutation responsible for chick albinism effects only the copper-containing tyrosinase system, but not the peroxidase system and that these depigmented chicks have granulocytes containing peroxidase.

In summary, the findings in our experiments, taken together with the work of others, indicate that ultra-violet irradiation of skin causes synthesis of new tyrosinase in melanocytes and that this enzyme becomes detectable in the Golgi apparatus and the

surrounding (connected) system of smooth endoplasmic reticulum. It seems likely that tyrosinase is then channelled from here via a tubular membrane system and becomes associated with the membrane of a focal dilatation of the smooth endoplasmic reticulum in which the earliest melanosomal structure has independently formed. Melanization of the structural protein can then take place and once this is completed, the connection with the tubular system is severed. Tyrosinase cannot be detected in the Langerhans cell, and there are no changes obvious in this cell after such a single dose of ultraviolet radiation.

b) Cellular responses to ultraviolet irradiation

The experiments described in detail in this Chapter were concerned with subcellular changes in dendritic cells after a single exposure of ultraviolet irradiation. Quantitative changes in the population of dendritic cells after ultraviolet irradiation is a separate issue and, as there are differences in opinion, the matter warrants some discussion.

There is no disagreement that there is proliferation and increased activity of melanocytes in response to ultraviolet irradiation (Pathak et al., 1965; Quevedo et al., 1965). An increase in dopa positive cells has been demonstrated conclusively at both light microscopic (Wolff and Winkelmann, 1967 and Szabo, 1967) and

ultrastructural (Mishima, 1967 and Zelickson and Mottaz, 1970) levels.

Ultraviolet induced changes in the Langerhans cell population is a more contentious issue, and the reason for the problem is not difficult to understand. Quantitative cellular measurements are best made at a light microscopic level as the size of the sample is much greater than that possible in electron microscopic studies. Accuracy depends on the specificity of the histochemical test used to label the cell being counted. Unfortunately the capricious nature of the histochemical demonstration of Langerhans cells in human epidermis makes quantitative studies based on most available reactions at least doubtful. Epidermal sheets from human skin are also much more difficult to interpret than those from the mouse or guinea pig where there are fewer layers of keratinocytes. For these reasons a quantitative electron microscopic approach has advantages, even though the sample is bound to be small.

Changes in the dendritic cell population in response to repeated ultraviolet irradiation were investigated by Zelickson and Mottaz (1968) and Zelickson et al. (1972) using a quantitative method of electron microscopy depending on 'linear scanning' (Carpenter and Lazarow, 1962) They duly confirmed an increase in the number of melanocytes but also noted a decrease in the number of Langerhans cells, which

were virtually absent in the specimens of skin studied after two weeks of daily ultraviolet irradiation. However the total number of Langerhans cells seen before (3) and after irradiation (0) were very small. Their results can be interpreted in many ways.

1) The sample was too small to come to any valid conclusion.
2) The melanocyte and Langerhans cell populations are in some way inversely related.
3) Ultraviolet irradiation effects not only the pigmentary (melanocyte) system, but also the keratinising function of the epidermis (Nix, 1964, and 1967) and changes in the Langerhans cell population may be related more to this.
4) Ultraviolet irradiation has a direct damaging effect on the Langerhans cell.

Linear scanning is an extremely time consuming procedure. Sample size is a problem and the change in actual numbers of Langerhans cells (from 3 to 0) may have been coincidental. Only larger additional studies, using the same method, will clarify the situation.

That the melanocyte and Langerhans cell populations are in some way inversely related has been suggested by the work of others.

Fan and associates (1959) noted a decrease in the number of gold chloride positively staining (Langerhans) cells in guinea pig epidermis after ultraviolet, X ray

and thorium X irradiation and attempted to relate this to the simultaneous increase in the number of dopa positive melanocytes. Breathnach et al. (1963) also noted a decrease in the number of gold-positive cells and an increase in melanocytes after the application of thorium X.

On the other hand Wolff and Winkelmann (1967) noted no difference in the number of A.T.P.'ase positive cells in guinea pig epidermis following daily ultraviolet irradiation even though there was a sharp increase in the melanocyte population.

Wolff (1972) has pointed out that the differences in the observations of the above groups may be more apparent than real. Different methods of irradiation (involving different wavelengths), different dosages, different species and different methods for the quantitative assessment of Langerhans cells could, inter alia, be responsible for the discrepancies.

Taking the results of the above studies into account and considering the findings in vitiligo and other depigmented conditions (Birbeck et al., 1961; Breathnach et al., 1965; Brown et al., 1967; Swanson et al., 1968 and Zelickson and Mottaz, 1968) it would seem that the problem is best answered by considering that melanocytes and Langerhans cells compete for the same territory in the basal layer (Breathnach et al., 1968; Riley, 1975). This concept

does not imply an inverse relationship between Langerhans cells and melanocytes and would fit in with the findings of Mishima, Kawasaki and Pinkus (1972) who noted an increase in the basal layer Langerhans cells in vitiligo but no increase in the total population.

Ultraviolet irradiation, as well as stimulating the melanocytes to divide and produce more pigment, effects other epidermal cells. There is epidermal thickening and desquamation (Nix et al., 1964 and 1965). It is possible that the decrease in number of Langerhans cells noted by the workers mentioned above is related primarily to keratinocyte changes rather than the melanocyte population. The fact that Lessard et al. (1968) were able to reduce the Langerhans cell population by about 80% with tape stripping of the skin, emphasises that some loss of Langerhans cells could be explained on this basis.

Direct ultraviolet damage of Langerhans cells is another mechanism that could explain their reduction after irradiation. However Langerhans cells appeared quite normal after ultraviolet irradiation of skin with a single dose of 6 X MED (page 97), and Langerhans cell damage has not been noted in sun-exposed vitiliginous skin and ultraviolet-irradiated patches of vitiligo (Zelickson and Mottaz, 1968).

Chapter V

THE EFFECT OF SUCTION INJURY TO THE SKIN

Including

1. INTRODUCTION

2. MATERIAL AND METHODS

3. RESULTS
   a) Light microscopy
   b) Electron microscopy

4. DISCUSSION

1. INTRODUCTION

Although stress due to suction is not commonly encountered in everyday life, both pressure and suction forces are exerted on the foot in ordinary locomotion. More topically, negative pressure may be exerted on the skin of aviators (especially astronauts) and divers in certain circumstances. Under experimental conditions, suction has also been used recently to evaluate dermo-epidermal adherence (Lowe and Van der Leun, 1968) and to monitor the state of blistering diseases, such as porphyria cutanea tarda (Copeman, 1970). In this Chapter the effects of suction on the epidermal dendritic cell population and keratinocytes are contrasted.

2. MATERIAL AND METHODS

Twenty-five volunteers were studied, of whom six were members of staff with no skin disease and the rest were patients with miscellaneous skin disorders (eczema, psoriasis, dermatitis herpetiformis and porphyria cutanea tarda). Blistering was produced on skin which was macroscopically normal, the flexor aspect of the forearm or the back of the hand being used. The suction chamber chosen was a modification of that described by Kiistala and Mustakallio (1967), and contained a perforated disc with seven apertures each of 5mm diameter. The negative pressure was

usually -150mm Hg but was occasionally -300mm Hg. Biopsies, with and without local anaesthesia, were taken before and 1, 5, 15 and 25 minutes after the onset of suction, as well as immediately after the appearance of a blister (70 - 100 min.).

Light microscopy

As outlined in Chapter II.

Electron microscopy

See Chapter II for routine details.

Lanthanum impregnation of tissue was carried out using the method of Hashimoto (1970). The specimens were fixed in 3% glutaraldehyde containing 1% lanthanum nitrate at 4°C for three days.

Altogether, 25 biopsies were processed and ten of these were examined electron microscopically. The results recorded are based on the study of numerous light microscopic serial sections and over 200 electron micrographs.

3. RESULTS

a) Light microscopy

After less than 1 minute of suction, appreciable changes were noted compared with control biopsies. There was slight oedema of the superficial dermis, and paranuclear vacuoles were seen in increasing numbers as the suction time increased; by 25 minutes nearly all the epidermal cells were affected (Plate 29), though there was no suggestion of dermo-epidermal separation. Some of the cells extending down follicular walls contained these distinctive vacuoles. They were invariably paranuclear, most often circular or ovoid in cross section, and frequently caused a crescentic deformation of the nuclear outline. The spatial relationship with the nucleus was variable. Even after 25 minutes of suction there was no evidence of spongiosis (extracellular oedema). Biopsies taken just after blister formation revealed dermo-epidermal separation, and PAS-stained sections showed that the positive staining basal lamina region remained on the topmost dermis (Plate 30), forming the blister floor. The cleavage line was frequently clear cut, but sometimes basal cells or dendritic cells (Plate 30) remained on the floor of the cavity. The roof of the blister was, therefore, formed by the total thickness of the epidermis. It usually, though not invariably, contained vacuolated cells and at this late stage spongiosis could sometimes be seen.

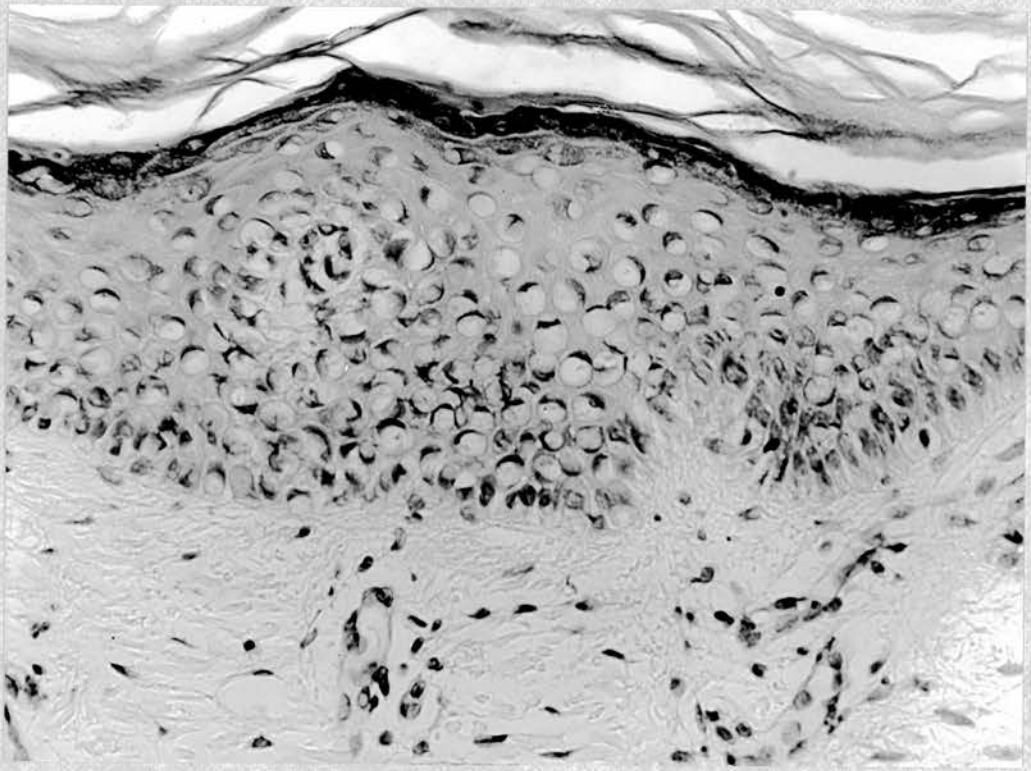


Plate 29 (X 300): Epidermis

25 min. -150 mmHg. Nearly all the keratinocytes contain large paranuclear vacuoles, and there is no sign of dermo-epidermal separation (H & E).

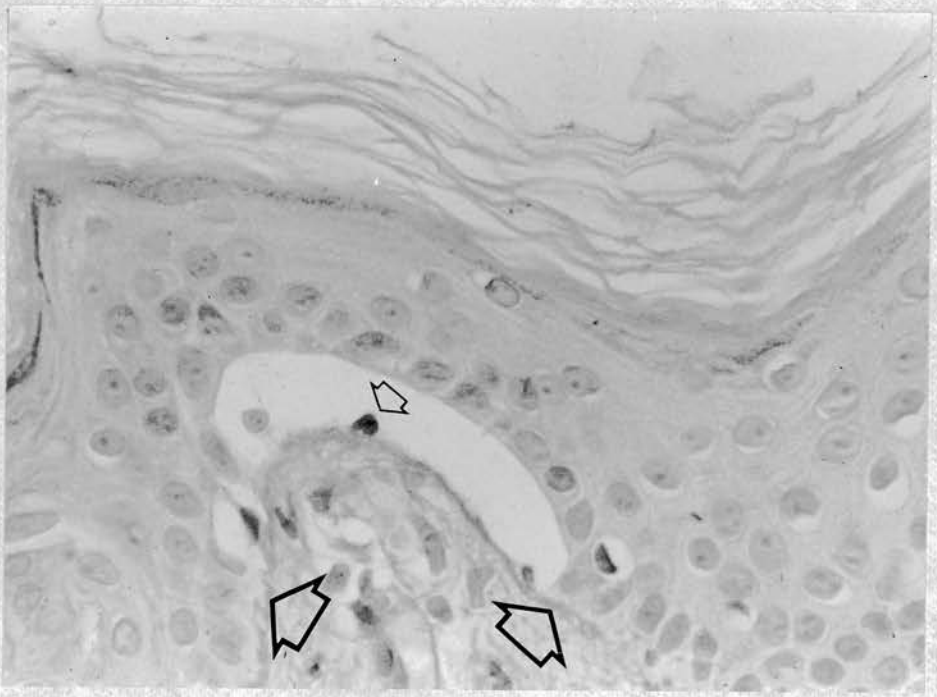


Plate 30 (X 720): Epidermis

135 min. -150 mmHg. An early blister forming by dermo-epidermal separation. The PAS positive staining basal lamina region remains on the floor (large arrows). A probable melanocyte (small arrow) has also been left behind on the floor (PAS).

b) Electron microscopy

(i) Keratinocytes

The vacuoles in these cells were quite characteristic and were seen in a few cells as early as 3 minutes after the onset of suction. They were bounded by a membrane (Plate 31), particularly during the earlier stages of their development, and in some instances the membrane could be resolved into a trilaminar structure. They rapidly achieved a diameter of up to 6μm, and when they reached this size they were invariably seen next to the nucleus, which they deformed into a half-moon shape (Plate 31). They were not within the perinuclear space, which appeared to be normal (Plates 31 - 33). Quite often a thin but distinct rim of cytoplasm could be seen between the outer nuclear membrane and the vacuolar membrane (Plate 31). They were often filled with a fine granular material that tended to accumulate towards the periphery of the larger vacuoles (Plate 31). With increasing suction, the vacuoles increased in size and their limiting membranes began to show breaks; their outlines became much less regular and melanosomes, melanosome complexes and membranous remnants could be seen within their cavity. At the blistering time, smaller membrane-bound vacuoles (diameter 80 - 160nm) could also be seen near the edges of the ruptured paranuclear vacuoles. At this late stage, the mitochondria showed degenerative changes. The distribution of the tonofibrils appeared relatively normal, though sometimes they were compressed by the larger vacuoles.

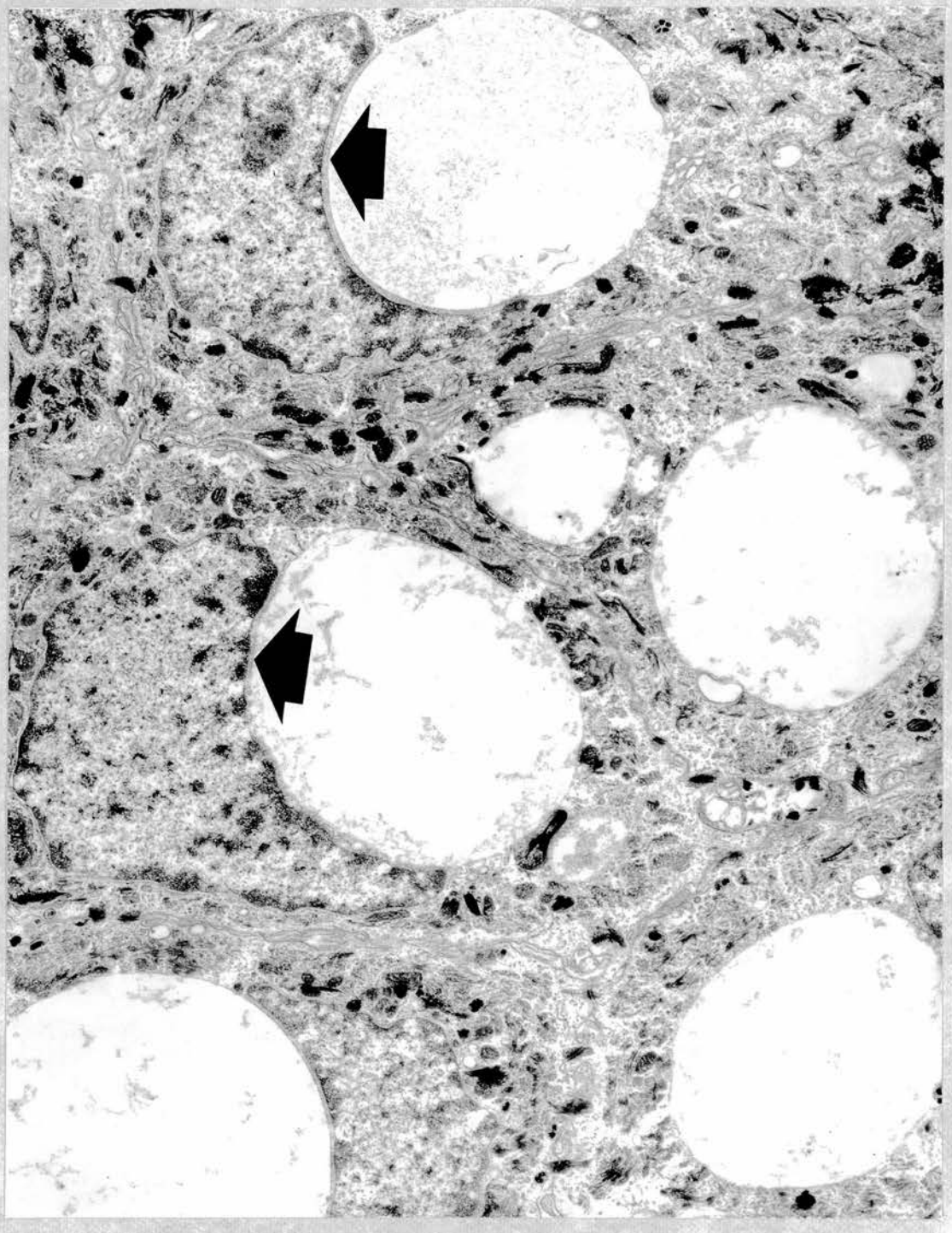


Plate 31 (X 12,500): Keratinocytes

25 min. -150mmHg. Preblister. Paranuclear vacuoles are prominent in this area. They deform the nuclei and contain varying amounts of granular material. There is a small rim of cytoplasm between the outer nuclear membrane and the vacuole membrane (arrows).

(ii) Dendritic cells

The paranuclear vacuoles, which were such a striking feature in keratinocytes, were not seen in either melanocytes or Langerhans cells. Frequently a dendritic cell, containing no paranuclear vacuoles, was seen surrounded by vacuolated keratinocytes (Plates 32 and 33). Preservation of the cellular contents of dendritic cells was also good, even in the late stages of blistering when keratinocytes exhibited considerable damage (Plates 34 and 35).

(iii) Intercellular space

This appeared normal at 25 minutes after the onset of suction, even though most cells contained paranuclear vacuoles. At the blistering time, however, a variable amount of intercellular oedema was seen, particularly at the blister edge, and in some places the distended space contained a fine granular material similar to that in the paranuclear vacuoles.

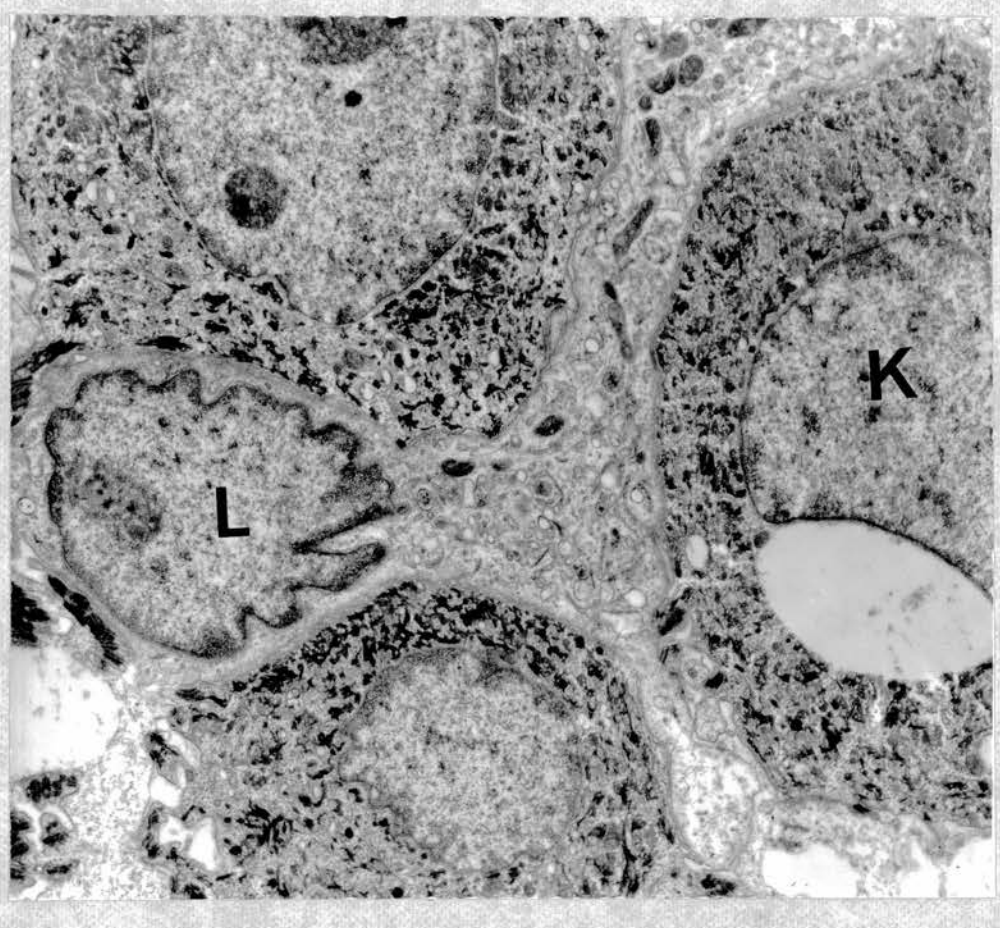


Plate 32 (X 10,000): Langerhans cell

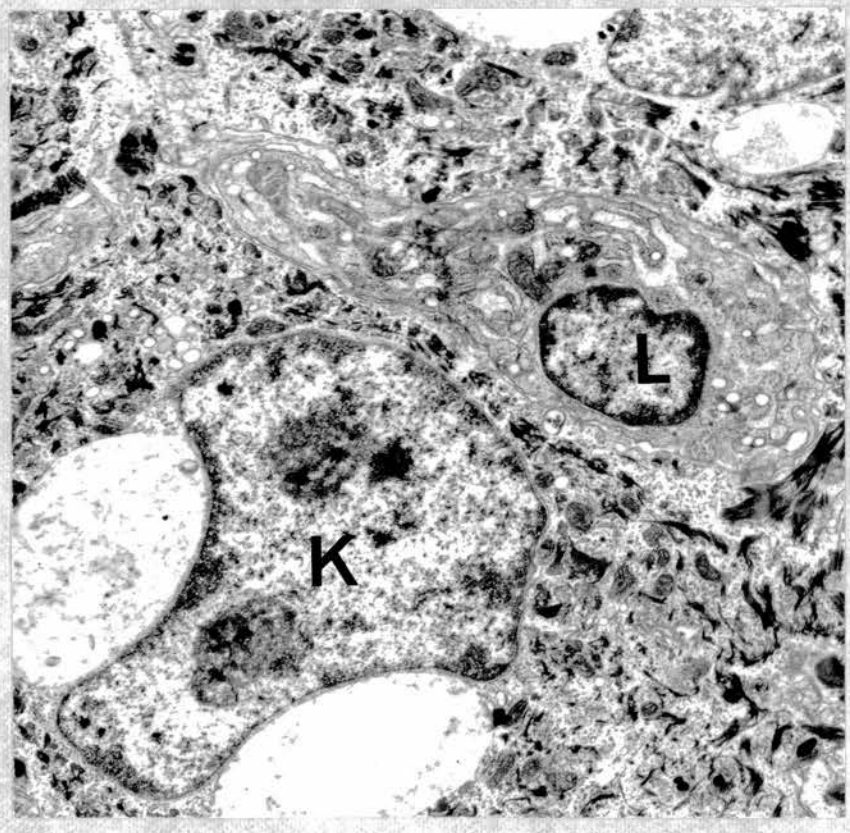


Plate 33 (X 11,500): Langerhans cell

In both plates vacuoles are seen in keratinocytes (K) but not in Langerhans cells (L). Both 90 min. -150mmHg.

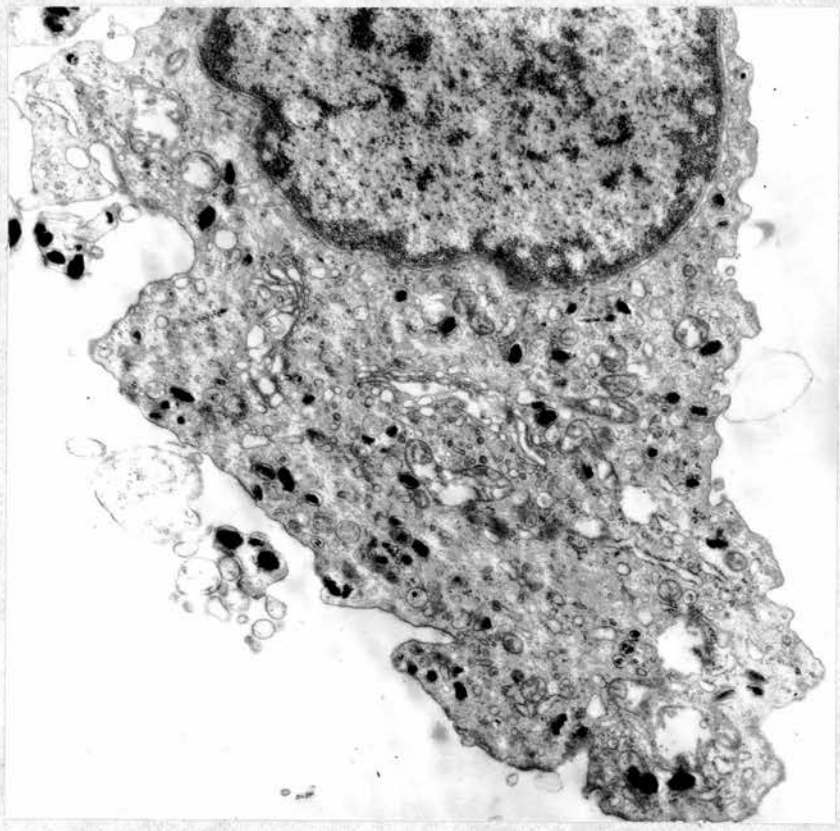


Plate 34 (X 13,000): Melanocyte and dendritic processes (arrows)
Cell in blister roof, dangling into cavity. Organelles are well preserved. (90 min. -150mmHg)

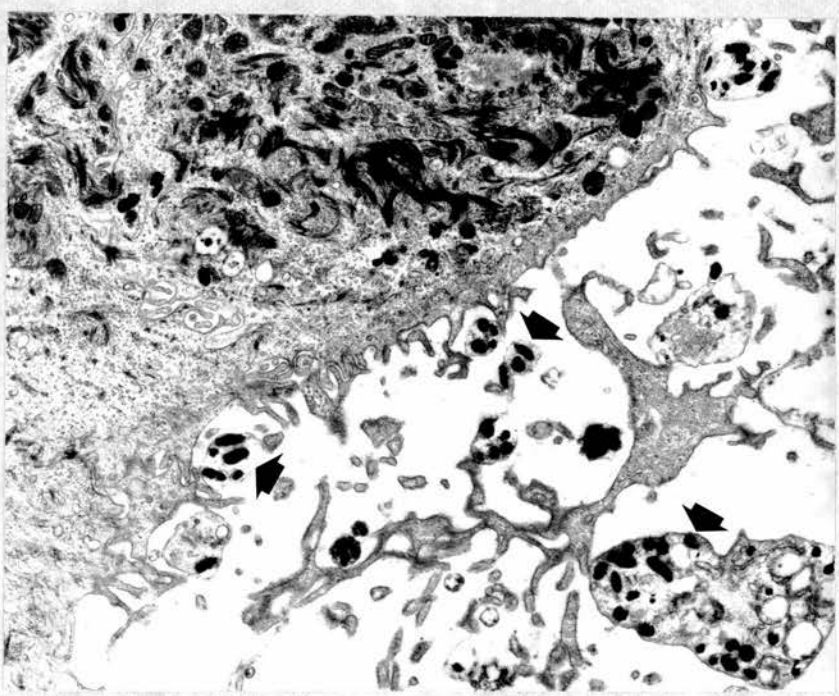


Plate 35 (X 11,000): Base of blister roof
Keratinocytes (top left) and dendritic processes of melanocytes (arrows) are well preserved. (90 min. -150mmHg)

(iv) Basal region

The blister was formed by dermo-epidermal separation. Cleavage was most often between the base of the basal cell and the basal lamina, so that the latter usually remained on the floor of the blister. However, fragments of the basal lamina were occasionally seen attached to the base of the basal cells in the roof, and sometimes the cleft occurred through the centre of basal cells. Keratinocytes and melanocytes were occasionally seen in the blister fluid (Plates 34 and 35) or lying on the floor of the blister.

Lanthanum impregnation

The intercellular space showed normal impregnation with lanthanum (Plate 36) and the tracer was never seen in the paranuclear vacuoles of keratinocytes (Plate 36).

A granule attached to a Langerhans cell wall was also impregnated with tracer, though the lanthanum did not stain a granule within the cytoplasm of the same cell (Plate 37).

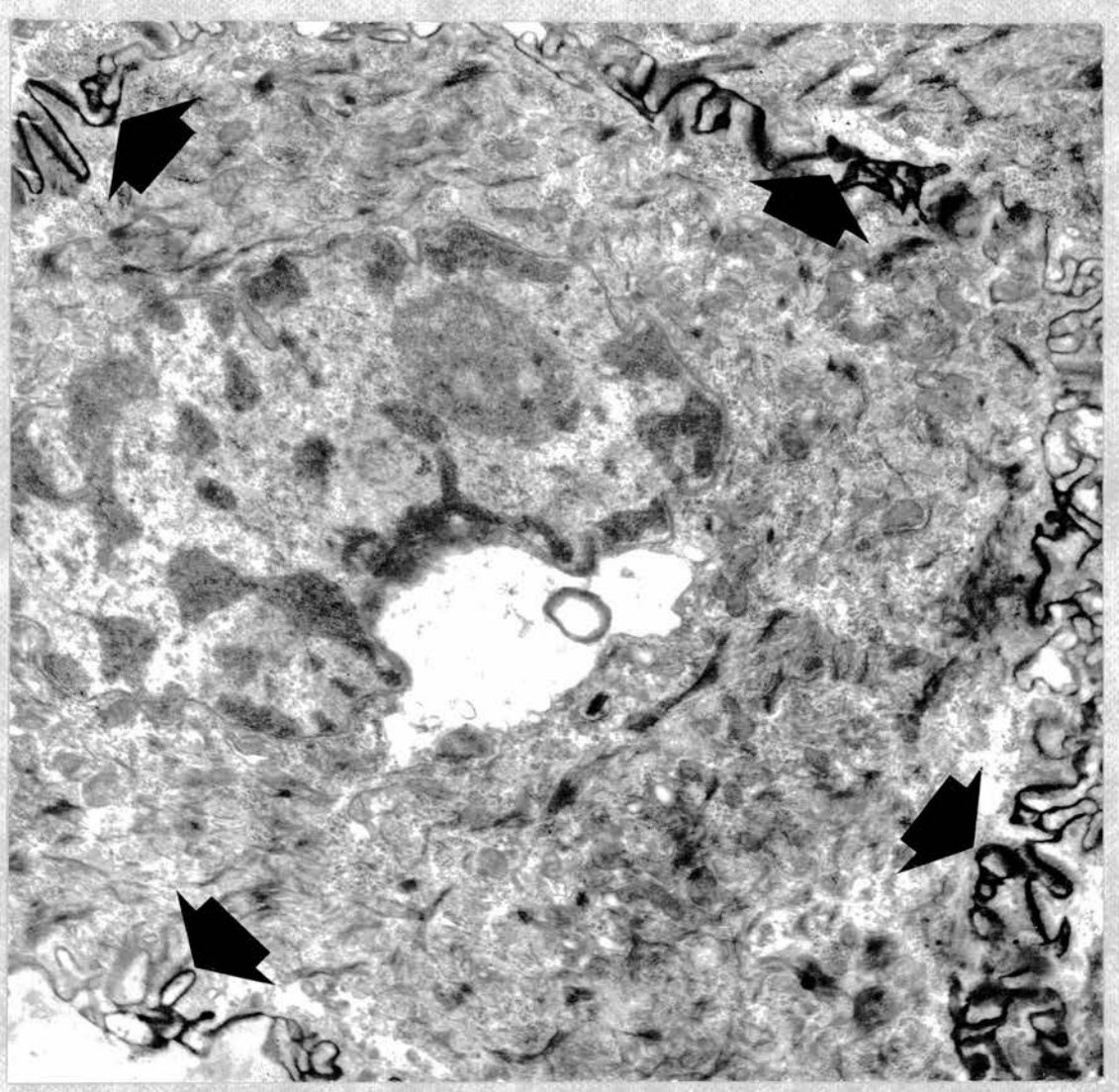


Plate 36 (X 18,000): Blister roof (95 min. -150mmHg)
Lanthanum (arrows) outlines the intercellular space around a keratinocyte containing a paranuclear vacuole, but there is no evidence of tracer in the vacuole.

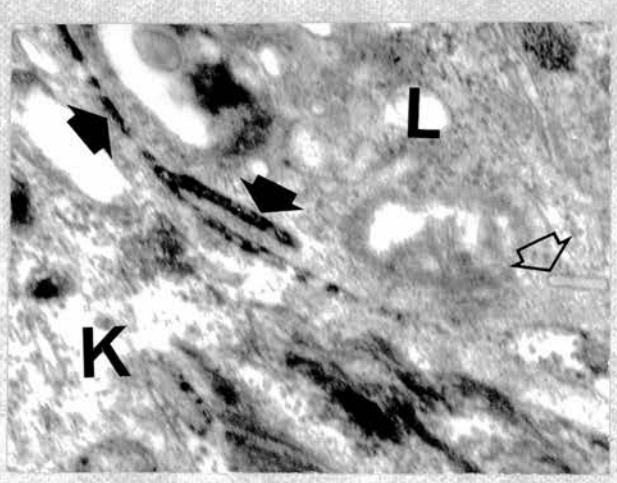


Plate 37 (X 25,000): Blister roof (95 min. -150mmHg)
Lanthanum impregnation. The lanthanum (arrows) stains the intercellular space and a Langerhans cell granule attached to the wall of the Langerhans cell L. Open arrow points to an unstained granule not attached to cell wall. Keratinocyte (K).

4. DISCUSSION

The nature and mechanism of formation of the paranuclear vacuoles present interesting problems. Similar vacuoles have been noted in human keratinocytes in dermographic weals (Cauna, Macy and Cralley, 1970), and have been produced experimentally by ultraviolet irradiation (Nix, 1965), by intradermal injection of hypertonic solutions of sodium chloride and of dextrose (Hönigsmann and Wolff, 1973), by suction (Copeman, 1970), and by the topical application of vitamin A to the human skin (Plewig, Wolff and Braun-Falco, 1971). Wolff and Hönigsmann (1973) believe that the vacuoles, seen in the study reported here, are identical to those produced by the intracutaneous injection of hypertonic solution into guinea pig skin. They have carried out extensive studies to determine the nature and mechanism of formation of the vacuoles. Using tracers such as colloidal silver and horse-radish peroxidase in the injected solution, they were able to show staining of both the intercellular space and the paranuclear vacuoles. The vacuoles were stained also with osmium soaking (a technique which outlines the endoplasmic reticulum) and did not contain ruthenium red (staining glycocalyx of cell surface) or nucleoside triphosphatase activity (seen commonly at the cell surface), suggesting that they were not formed by infolding of the cell membrane. They concluded that there was a canalicular system of interconnected cisternal

channels within keratinocytes, which formed a direct communication between the extracellular and the perinuclear spaces, and that it could unfold under certain experimental and pathological conditions (Hönigsmann and Wolff, 1973). In this study, the development of the vacuoles was too quick for endocytic mechanisms to be considered as a cause for the uptake of the fluid. The rapidity of their formation suggests also that fluids are sucked from the intercellular space into pre-existing cavities along communications of endoplasmic reticulum, as suggested by Hönigsmann and Wolff.

The precise role of lanthanum in staining the cell surface is uncertain. Doggenweiler and Frenk (1965) considered that it stained lipids of the cell surface, while Behnke (1968) and Shea (1971) felt that its staining was specific for the demonstration of acid mucosubstances. Its delineation of the extracellular space seems to be due to non-specific precipitation (Revel and Karnovsky, 1967). It has been used to outline the extracellular space in human epidermis (Wolff and Schreiner, 1968), and to show continuity between the extracellular space and granules attached to the walls of Langerhans cells (Hashimoto, 1970). In this study, it was at first surprising to note that there was no sign of lanthanum penetration into the paranuclear vacuoles within keratinocytes. The most likely

explanation of this would seem to be that a communication, like that envisaged by Hönigsmann and Wolff, is patent only during the period of suction. As this communication cannot be penetrated by lanthanum in normal skin (Wolff and Schreiner, 1968), Hönigsmann and Wolff have tentatively suggested the presence of a valve or sluice-like mechanism which opens only under certain circumstances, e.g. when in contact with hypertonic solutions or an increased flow of intercellular fluid. If this were so, the lanthanum, used only during fixation of the biopsy specimen, would not be expected to gain access to the postulated canalicular system of communication.

Although ultrathin serial sections were not made, the fact that paranuclear vacuoles were not seen in melanocytes or Langerhans cells seemed significant. Examination of numerous sections revealed no affected dendritic cells, even though neighbouring keratinocytes contained large paranuclear vacuoles. It should also be pointed out that neither Cauna et al. (dermographic weals) nor Hönigsmann and Wolff (intradermal injection of hypertonic solutions) mentioned such vacuoles in epidermal dendritic cells, in spite of their obvious presence in keratinocytes. There are two likely explanations. First, it is possible that communications between the extracellular space and the system of endoplasmic reticulum, demonstrated in keratinocytes (Hönigsmann and Wolff, 1973), do not exist in dendritic cells. Secondly, it is conceivable that keratinocytes.

because of their intercellular connections (desmosomes and intertwining villous processes, seen in Plate 4) are more prone to the effects of suction than dendritic cells, which merely move as a whole in the direction of the suction force.

Chapter VI

THE EFFECT OF FRICTION INJURY TO THE SKIN

Including

1. INTRODUCTION

2. MATERIAL AND METHODS

3. RESULTS
   a) Light microscopy
   b) Electron microscopy

4. DISCUSSION

1. INTRODUCTION

For gripping and for moving, man cannot function without friction between him and his environment. However if this stress is prolonged or unusually severe, pathological changes occur, culminating in blisters and erosions. Human epidermis subjected to frictional stress has been studied by the light microscope (Naylor, 1955; Fukuyama and Cortese, 1968), and by autoradiography (Epstein, Fukuyama and Cortese, 1969), but no electron microscopic studies have been published. In this Chapter the effects of friction on the epidermal dendritic cell population and keratinocytes are contrasted.

2. MATERIAL AND METHODS

Six volunteers with ages ranging from 20 to 78 years were studied. Four had miscellaneous skin diseases (psoriasis, 3 and tinea pedis, 1). Macroscopically normal skin on the dorsal surface of the forearm was chosen for the experiments. The apparatus used was similar to that described by Naylor (1955), and consisted of a machine which rubbed skin with a reciprocating action at measurable speeds and forces until a blister or its immediate consequence, an erosion, was produced. The head was hemispherical and made of stainless steel. A description of the machine and its operation has already been published

(Comaish, 1973). The friction force varied from 1.9 to 3.9 Newtons* and blistering occurred at times ranging from 5 to 14 minutes. Two of the subjects agreed to having biopsies taken from four different sites, allowing sequential changes to be studied up to the time of an erosion (e.g. biopsies at 1, 5, 10 and 13 minutes after the onset of friction).

Light and electron microscopy

The techniques used were the same as those in Chapter V.

Altogether 14 biopsies were taken. Ten were prepared for light microscopy and were serially sectioned, and the remaining 4 (2 taken just after blister formation and 2 approximately midway between the onset of friction and expected blister time) were processed for electron microscopy.

3. RESULTS

No significant difference was noted between different subjects, and the results were therefore grouped as a whole. They are most conveniently summarized by describing changes seen at the time of blistering (e.g. 14 minutes) and those seen much earlier (e.g. 5 minutes), where the biopsy was taken from intact but slightly erythematous skin. The changes

* 1 Newton (N) causes an acceleration of unity (1 metre per second) to a 1 kilogram mass.

in the latter (hereafter described as 'early' changes) were more subtle and could often be traced only by searching through numerous serial sections with light microscopy, and by carefully checking thick sections embedded in Araldite before cutting ultrathin sections for electron microscopy.

a) Light microscopy

(i) Early changes

The earliest changes were noted in the upper squamous cell layer (Plate 38), whilst the overlying horny and granular cell layers were relatively unaffected. The changes were quite frequently noted adjacent to hair follicles and sweat duct orifices, and consisted of a localized area of eosinophilic change in the haematoxylin and eosin sections (between asterisks in Plate 38). The cells showed marked intracellular oedema, their margins were indistinct, and there was some spongiosis. Small vesicles could be seen when the changes were more developed (Plate 38). The superficial dermis showed only mild oedema.

(ii) Late changes

Blistering occurred intra-epidermally at the level of the upper Malpighian layer (Plate 39). Many of the neighbouring epidermal cells in the floor and to the side of the blister showed changes similar to those seen in the early stages and described above.

These sometimes extended to the level of the basal cells (arrow, Plate 39), but the dermo-epidermal junction remained intact. However, below the blister the basal lamina region stained less intensely with PAS (Plate 39). The superficial dermis still showed only mild oedema.

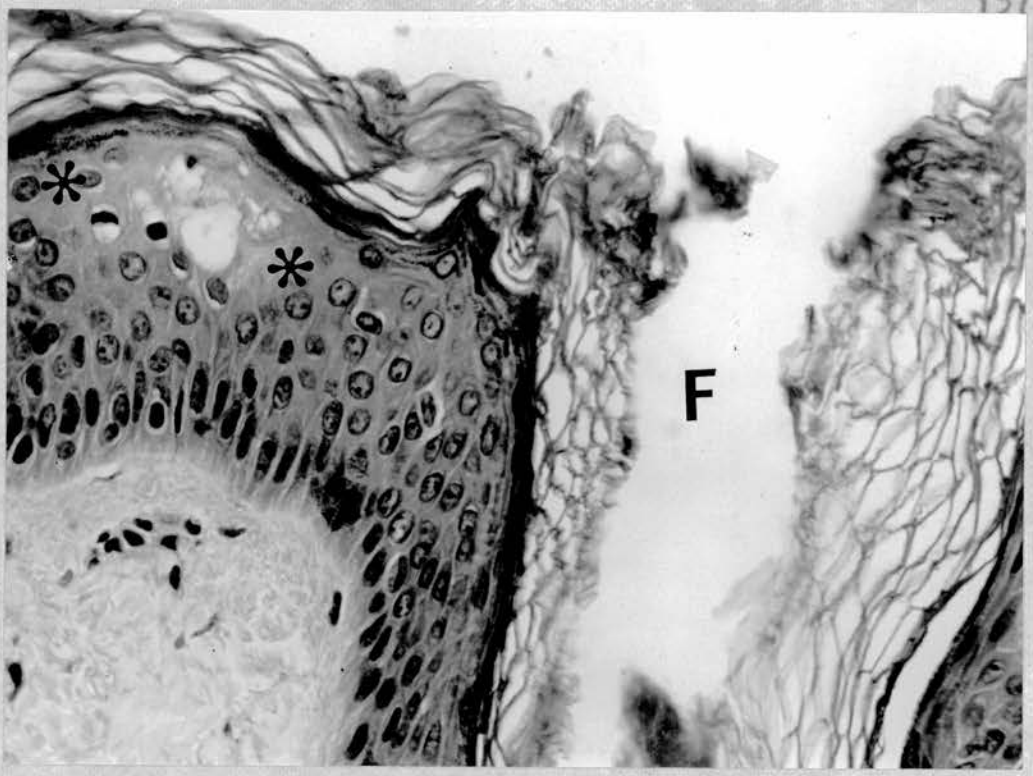


Plate 38 (X 420): Early changes (5 min. rubbing, 2.6N) are seen between the markers just deep to the granular cell layer. F, follicle (H & E).

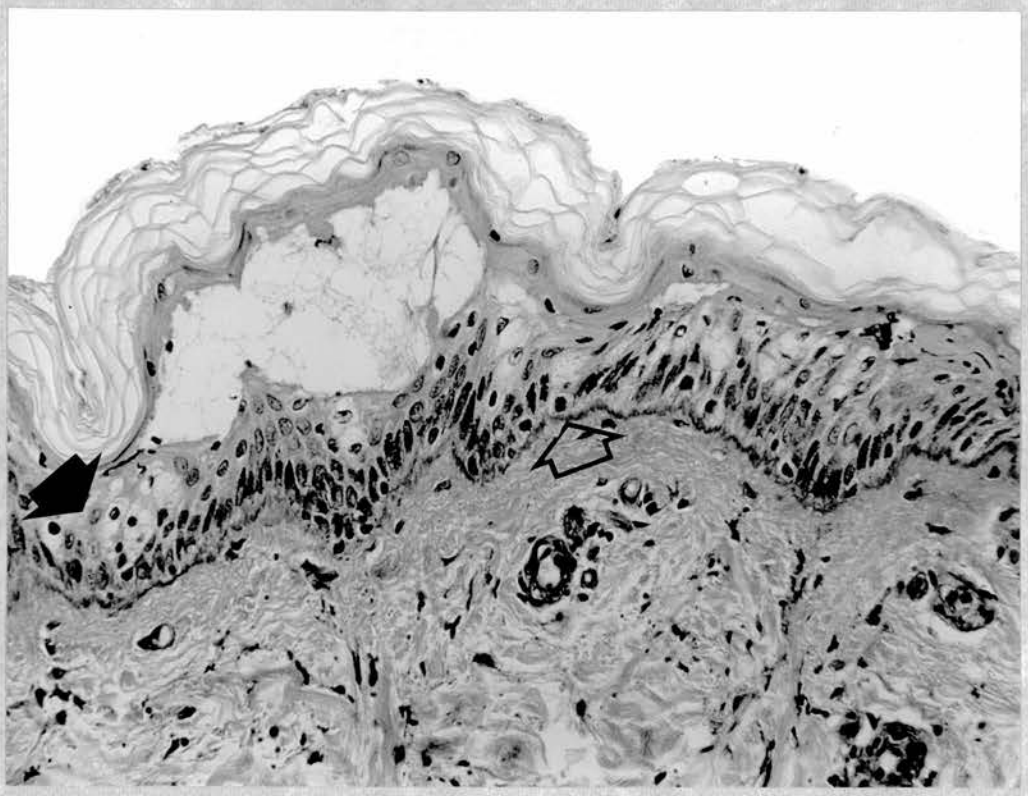


Plate 39 (X 270): Late changes (14 min. rubbing, 2.0N) show an established intra-epidermal blister in the upper Malpighian layer. Broad arrow points to cells in base which show marked intracellular oedema. The basal lamina (open arrow) shows some lack of definition below the blister but is otherwise intact (PAS).

b) Electron microscopy

(i) Early changes (Plates 40, 42 and 43).

Plates 40, 42 and 43 are representative of the early changes seen in areas such as that marked in Plate 38. There was obvious intracellular oedema (arrows, Plate 40), which was most noticeable at the cell periphery, and when severe this produced a granular appearance in an area devoid of organelles. As a result of this, the tonofilaments sometimes appeared clumped, resembling necklaces around the nuclei (Plate 40). In places, small vacuoles (approximately 0.8μm in diameter) appeared in the oedematous periphery of keratinocytes, and the membranes of these cells were often ruptured (V_1, Plate 40), allowing their granular contents to spill into small dilatations of the extracellular space (V_2 and V_3, Plate 40). The dendritic cells appeared to be relatively unaffected.

(ii) Late changes (Plate 41)

Similar though more pronounced changes were seen around established blisters. Plate 41 is representative of changes seen in an area adjacent to the arrow in Plate 39. The intracellular oedema, again most marked at the periphery of keratinocytes, was increased and the extracellular space showed numerous localized dilatations which frequently contained a granular

material (arrows, Plate 41). The cellular organelles were damaged and distorted, and on a very few occasions vacuoles (approximate diameter 0.3μm) were seen beside keratinocyte nuclei. They were much smaller than those seen in the previous suction experiments and never appeared to cause nuclear deformation. Some desmosomal attachments were still evident (open arrows, Plate 41), though membranous rupture and intracellular oedema often made identification of the cell borders difficult. The large intraepidermal blisters contained in places a granular material devoid of organelles.

Once again little change was noted in the dendritic cells even though surrounding keratinocytes were severely damaged (Plates 41 and 43).

Lanthanum staining

The tracer outlined both the extracellular space and its damaged dilatations (Plates 42 and 43). As in the suction experiments no impregnation of granules within Langerhans cell was seen even though the tracer often filled the space around such cells (Plate 42).

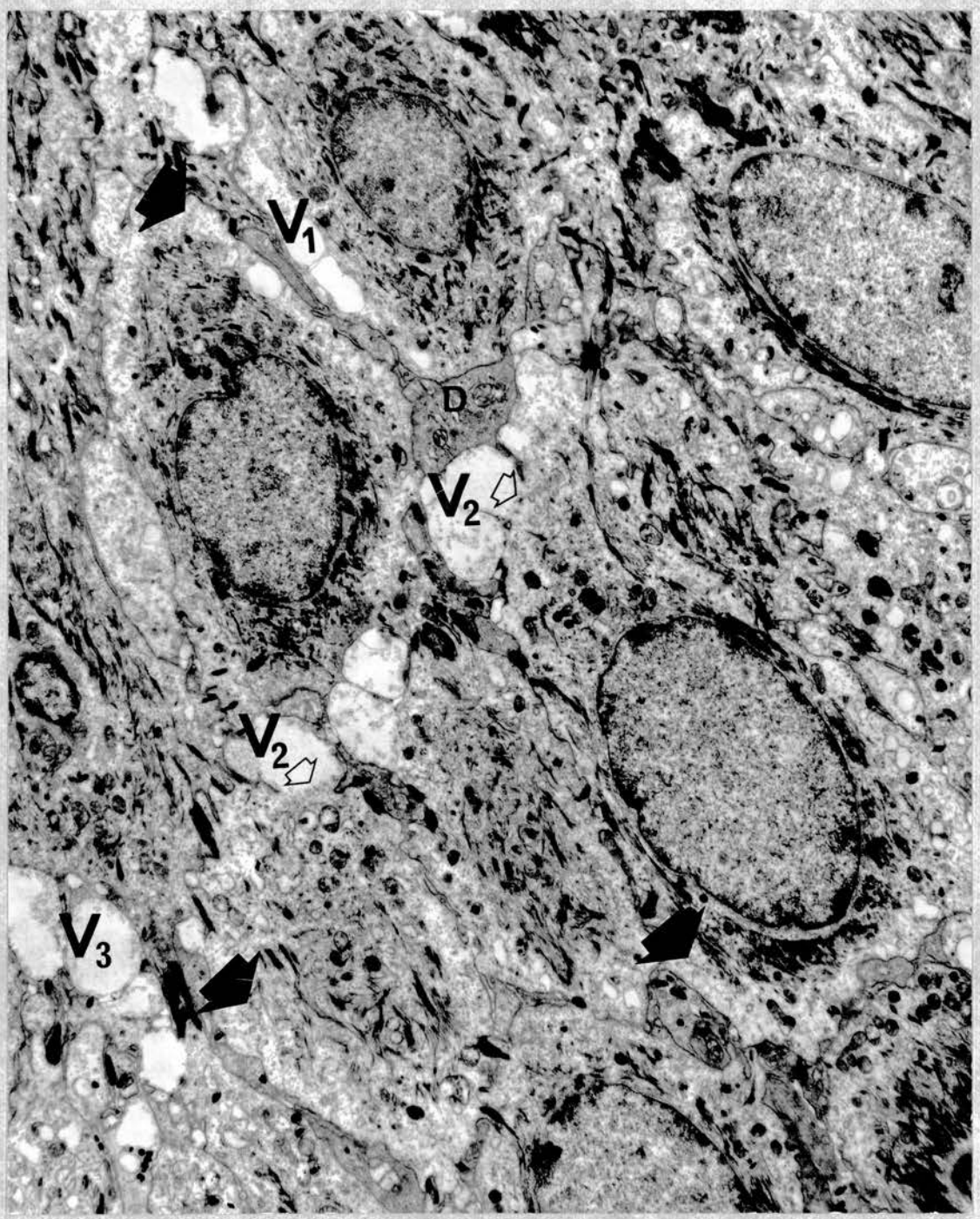


Plate 40 (X 7,000): Early changes (5 min. rubbing) seen in an area similar to that indicated in Plate 38. The keratinocytes show marked intracellular oedema (broad arrows) and the cell peripheries are relatively devoid of organelles. Open arrows point to places where the membranes are ruptured. V1, membrane bound vacuoles in the periphery of the cell; V2, vacuoles in extra-cellular space containing granular material adjacent to ruptured membranes; V3, extra-cellular space dilatations; D, dendritic cell process.

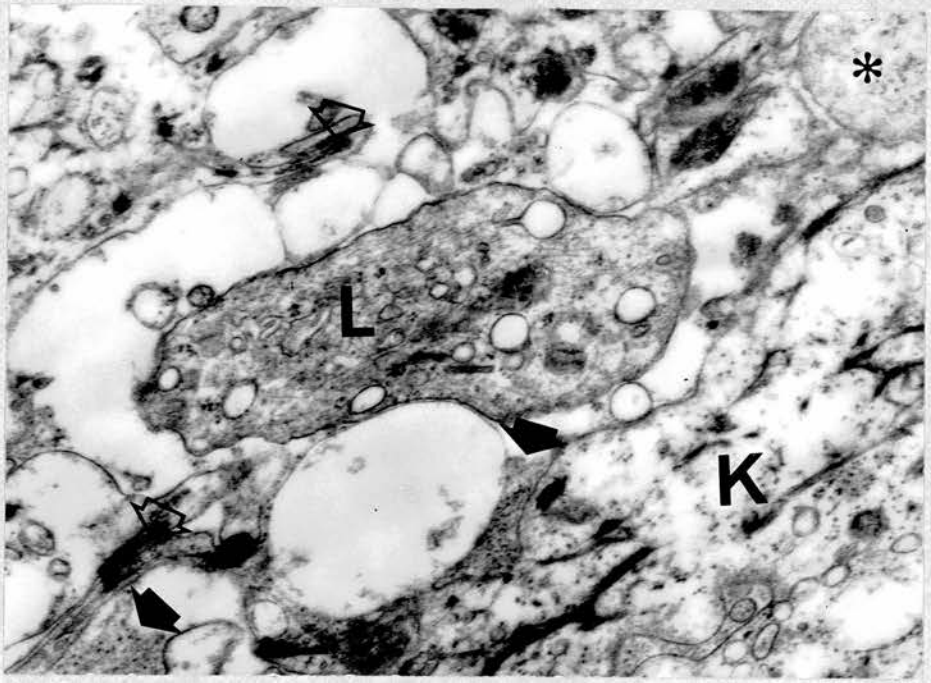


Plate 41 (X 27,000): Langerhans cell

Late changes (6.5 min. rubbing) at the edge of an erosion seen in an area similar to that indicated in Plate 39. There are numerous dilatations of the extracellular space and some contain a granular material (arrows) similar to that seen in degenerative cells (*) and oedematous keratinocytes (K). The process of a Langerhans cell (L) appears unaffected. Open arrows point to intact desmosomes.

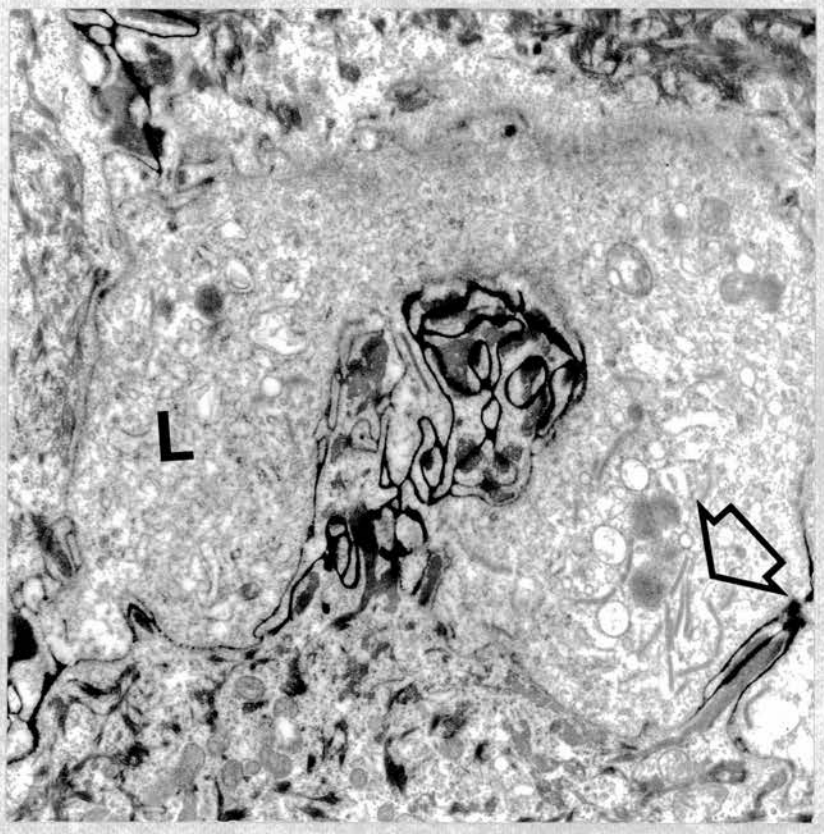


Plate 42 (X 13,000): Langerhans cell (L) surrounded by lanthanum. Note no lanthanum in granules (arrow) within cell and good preservation of intracellular organelles. (5 min. rubbing)

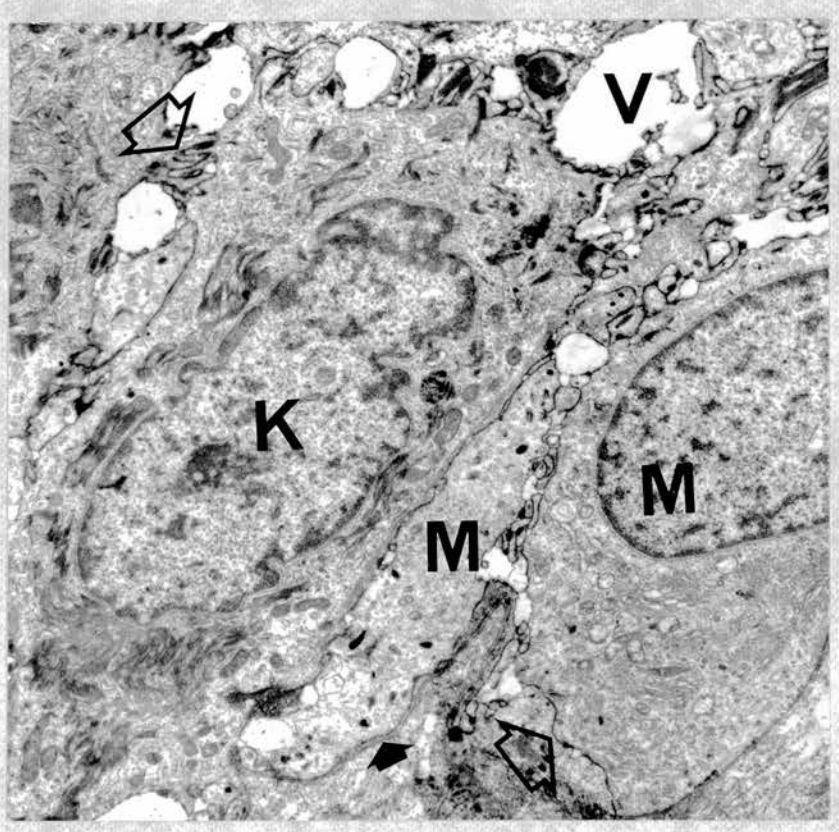


Plate 43 (X 8,000): Basal cell layer. Early changes (5 min. rubbing) are also seen in the extracellular space near the dermo-epidermal junction. Lanthanum (open arrows) outlines the space and its localized dilatations (V). M, melanocyte; K, keratinocyte; small arrows point to basal lamina.

4. DISCUSSION

Epidermal injury due to friction is clearly different from that produced by suction. The cell membranes and intercellular space bear the brunt of frictional stress. There is keratinocyte intracellular oedema, rupture of membranes and the pouring out of cell contents into the intercellular space, which becomes irregularly dilated. Eventually the damaged cells become necrotic and small intraepidermal blisters are formed.

Mild to moderate thermal burns produce appearances similar to those observed above with friction trauma. In both there are intracellular oedema, early loss of organelles, clumping of tonofilaments, and leakage of cytoplasm through ruptured plasma membranes into the intercellular space (Pearson, 1965). Nevertheless, caution should be exercised before inferring that friction damages cells by a local rise in temperature. Naylor (1955), by varying the speed of rubbing and by using heads of different thermal conductivity, concluded that the damage was not temperature-dependent, but more likely due to mechanical distortion of the cells. The observations reported here are quite consistent with such a view.

Once again the dendritic cells appear to escape major damage. Presumably the predominantly basal cell layer localization of melanocytes protects them from

damage which is more noticeable higher up in the epidermis (Plates 38 and 39). The mechanical distortion of keratinocyte membranes is also likely to be greater because of their complex intercellular connections (desmosomes and intertwining villous processes). This is in sharp contrast to the dendritic cells which, because of their lack of intercellular connections, are more malleable, and therefore have their own built in shock absorber system.

Chapter VII

HISTIOCYTOSIS X

Including

1. INTRODUCTION

2. MATERIAL AND METHODS

3. CASE REPORT
   a) History
   b) Examination
   c) Investigations
   d) Progress
   e) Autopsy
   f) Light microscopy
   g) Electron microscopy

4. DISCUSSION

1. INTRODUCTION

Histiocytes are macrophages of connective tissues. They belong to the mononuclear phagocyte system (Langevoort et al., 1970) which comprises all highly phagocytic mononuclear cells and their precursors. In this Chapter the word 'histiocyte' is preferred to 'macrophage' because of the presently accepted terminology of the proliferative disorders of this cell ('histiocytoses' and 'histiocytosis X').

Rappaport (1966) classifies the histiocytoses (reticuloendothelioses) as follows.

1) Reactive histiocytoses. Reactive histiocytic proliferations that may occur in response to known infections (e.g. typhoid, brucellosis and miliary tuberculosis) or other disease processes (e.g. haemolytic anaemia.)
2) Malignant histiocytoses. The systemic proliferation of neoplastic, morphologically malignant histiocytes and their precursors.
3) Differentiated histiocytoses. The systemic proliferation of differentiated histiocytes, which lack the cytological abnormalities of neoplastic cells, but are invasive and are of unknown aetiology.

In 1953, Lichtenstein, using histopathological arguments, grouped the differentiated histiocytoses, Letterer Siwe disease, Hand Schüller Christian disease and eosinophilic granuloma under the name of histiocytosis X,

the letter "X" denoting aetiological ignorance of the conditions.

In 1965, Basset and her colleagues noted Langerhans cell granules in the cells of a pulmonary eosinophilic granuloma, and since then similar granules have been found in Letterer Siwe disease and Hand Schüller Christian disease (see Chapter I).

Histiocytosis X is a rare disease. In the last five years I have been able to obtain pathological material from only one such patient even though my interest in the condition is known by the paediatricians in Edinburgh. Although there was nothing particularly unusual about this case, details (especially the pathological features) are presented below as they illustrate well the findings of Madame Basset. The ultrastructural appearance of the cells containing Langerhans cell granules will be contrasted with that of the cells in malignant melanoma, which will be considered in much greater detail in Chapter VIII.

2. MATERIAL AND METHODS

As outlined in Chapter II.

3. CASE REPORT

a) History

T.C., a thirteen month old baby girl was admitted to the Royal Hospital for Sick Children (August 1973) with broncho-pneumonia. There had been an eight day history of cough, and respiratory distress became progressive. The infant had failed to gain weight satisfactorily in the previous five months, and four months prior to admission her mother had noted scaly and crusted lesions in the scalp. The infant's lower gums had become bluish and swollen some three months before admission. They had bled occasionally though a dentist had attributed the appearance to teething.

b) Examination

Examination revealed the following abnormal findings.

(i) Marked respiratory distress (respiratory rate 80/ minute), but not cyanosed on breathing air. Indrawing of soft tissues.

(ii) A few crusted pink papules, about 2 - 3mm in diameter, extending over the posterior scalp and nape of neck. Isolated individual lesions were seen on the mid back and anterior chest. Removal of the crust revealed a glistening pink papule which became purpuric when rubbed (Plates 44 and 45). There was crusting of both external auditory canals.

(iii) The gums were swollen, bluish and purpuric in places. There was some crusting but no bleeding. The mucous membrane of the upper lip was ulcerated and crusted (Plate 46).

(iv) The infant was two standard deviations below the mean in height and weight.

Interestingly, auscultation of the chest was normal, and there was neither hepato-splenomegaly nor lymphadenopathy.

Plate 44: Histiocytosis X. Crusted scalp lesions.

Plate 45: Histiocytosis X. Solitary papule on chest.

Plate 46: Histiocytosis X. Swollen purpuric lower gum and ulcerated upper lip.

c) Investigations

Analysis of arterial blood gases confirmed the clinical evidence of respiratory failure, and the p CO_2 began to rise in spite of controlled oxygen therapy.

The chest X-ray revealed extensive bilateral cystic changes in the lungs with associated broncho-pneumonia.

The haemoglobin, white cell count and differential platelet count and standard liver function tests were normal.

Biopsy of the skin lesions showed a picture typical of histiocytosis X of the Letterer Siwe type (see later).

d) Progress

There was steady deterioration in the infant's condition, and in spite of symptomatic oxygen therapy and treatment with prednisolone and vinblastine, she died on her ninth day in hospital after developing two tension pneumothoraces.

e) Autopsy

This confirmed the diagnosis of histiocytosis X of the Letterer Siwe type. There was extensive histiocytic infiltration of the lungs and each of the abnormal cystic spaces were lined by histiocytic cells. The skin and gum lesions were characteristic of

histiocytosis X, and the thymus was virtually destroyed by the histiocytic cell proliferation.

Sections of lymph nodes, liver, spleen, pancreas, adrenal gland, pituitary, bone marrow (skull), kidney, stomach, ileum, uterus and ovary showed no pathological features.

f) Light microscopy of skin lesions

Biopsies from two crusted papules (nape of neck and back) revealed a picture typical of that seen in Letterer Siwe disease. Plate 47 (from back lesion) is a low power view showing the dense histiocytic accumulation in the upper dermis. It has a fairly well demarcated lower margin, and pushes up against the epidermis which is thinned and shows obliteration of its rete pegs. In places, cellular invasion of the epidermis and crust formation on the surface can be seen. No cytological detail is evident at this power, and although extravasation of red blood cells in the infiltrate was pronounced, it cannot be recognized here.

Plate 48 (from nape of neck) shows a close up of the infiltrate in the papillary dermis. The infiltrate consists of monomorphic histiocytes with oval or slightly indented nuclei and plentiful mildly eosinophilic cytoplasm. Mitotic figures are not seen here (and are usually very rare) and cellular atypia is slight. In some areas the cells are loosely aggregated and separated

by oedema. Occasional multinucleated histiocytes are seen. There is invasion of the epidermis with formation of foci of histiocytes underlying a surface crust.

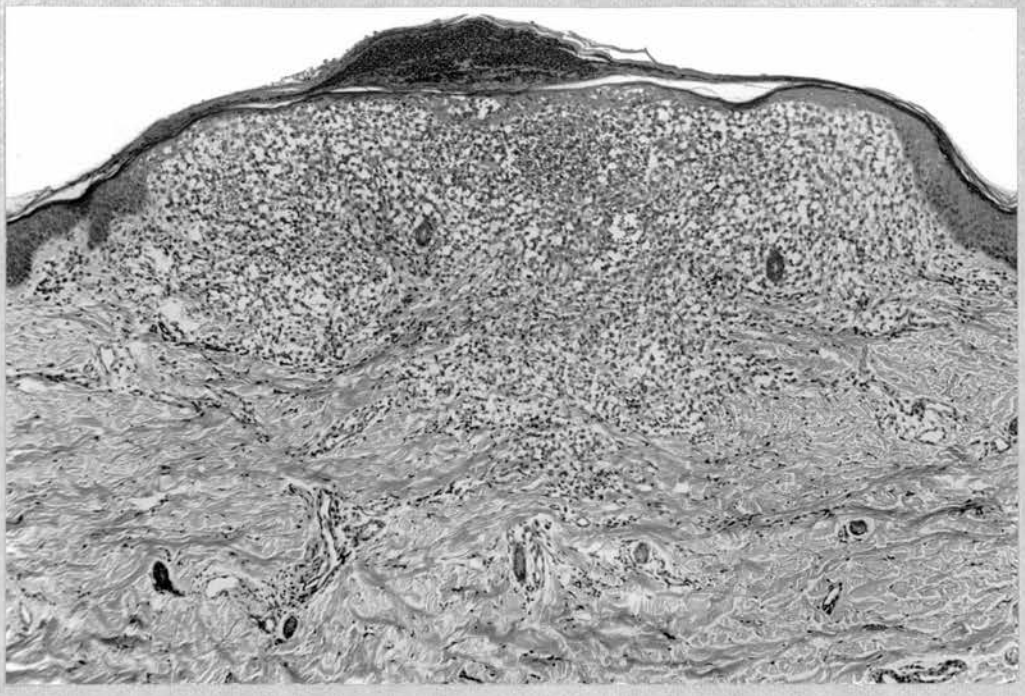


Plate 47 (X 75): Histiocytosis X. Lesion on back. Localized upper dermal accumulation of histiocytes, with epidermal thinning and invasion. (H and E)

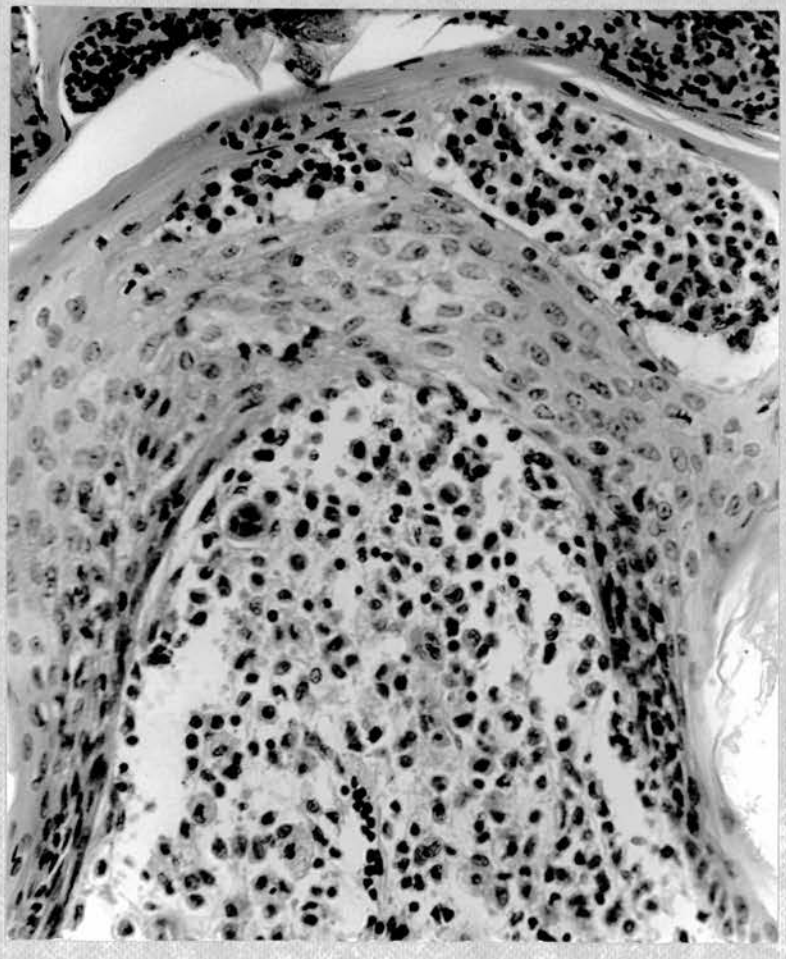


Plate 48 (X 340): Histiocytosis X. Abnormal histiocytes and giant forms in the papillary dermis. Epidermal invasion by infiltrate. (H and E)

g) Electron microscopy (skin lesion from back)

The infiltrate consisted mainly of histiocytes with a few scattered lymphocytes and free red blood cells.

The histiocytes could be divided into two types according to their ultrastructural characteristics.

(i) Undifferentiated histiocyte (Plate 49)

Cells of about 15μm diameter which had a ruffled and villous plasma membrane. Their nuclei were slightly convoluted and the nucleoli normal. Their cytoplasm contained no specific organelles but was often crowded with mitochondria, and free ribosomes and rough endoplasmic reticulum were frequently prominent. Some cells contained numerous lysosomal bodies, phagosomes and fat droplets.

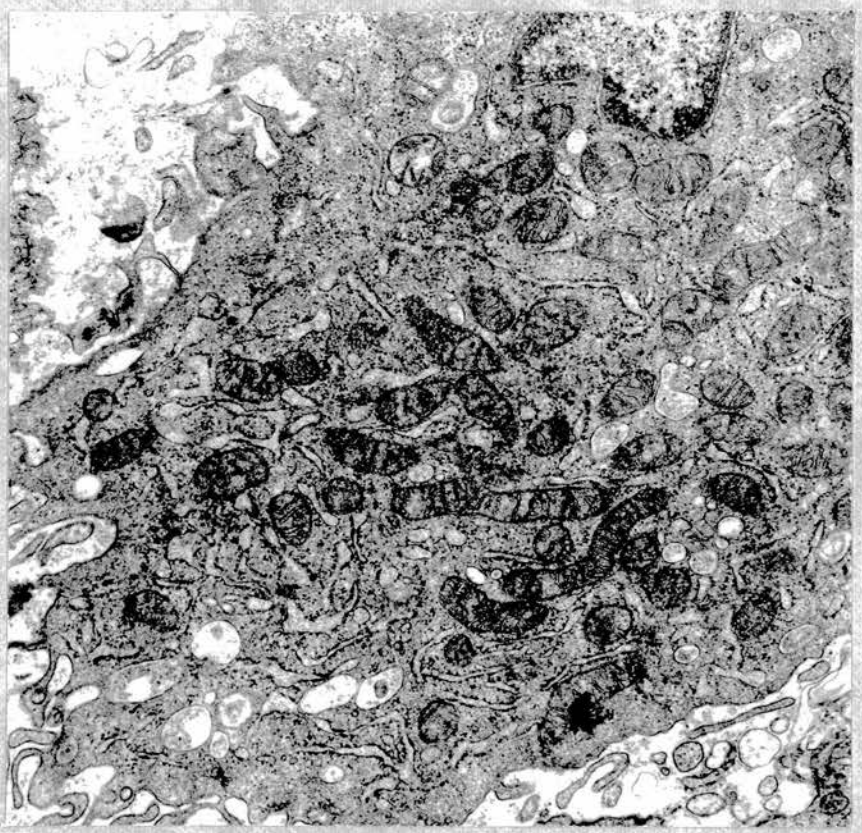


Plate 49 (X 18,000): Histiocytosis X. Part of an undifferentiated histiocyte in the infiltrate.

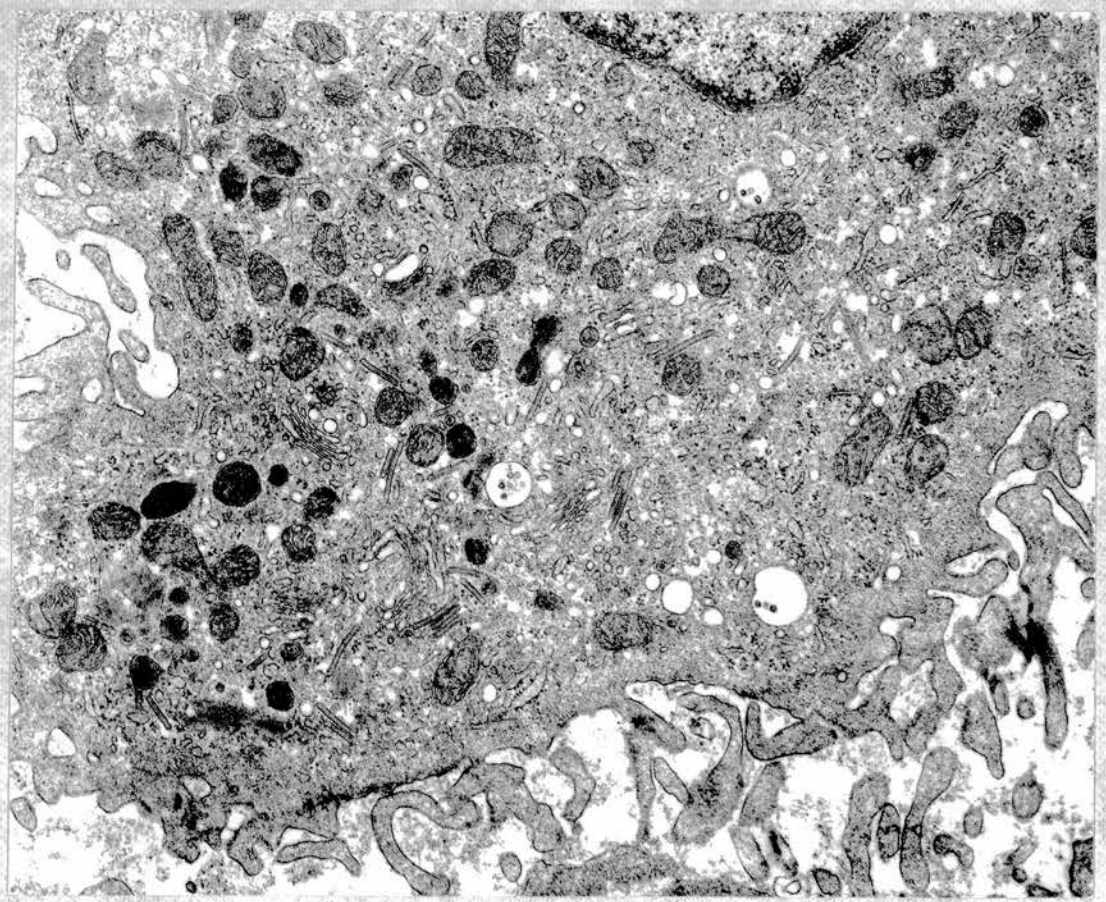


Plate 50 (X 18,000): Histiocytosis X. Part of a cell with many Langerhans cell granules. Note numerous villi at cell membrane.

(ii) Cells with Langerhans granules

About one in three cells of the infiltrate contained Langerhans cell granules (Plates 50 - 52). The picture was most striking. The cells were about 10μm in diameter and had slightly convoluted nuclei, but normal nucleoli. Their plasma membranes were again ruffled and showed numerous villous processes. Many were literally packed with Langerhans cell granules (Plates 51 and 52), which appeared much more plentiful than in normal epidermal Langerhans cells. The granules also showed more exaggerated profiles than are seen in normal epidermis. The measurements of the rod like structures were:

distance between external walls - 400 A°

periodicity of central lamella - 115 - 130 A°

length - variable, but up to 1μm.

Large vesicles, with a fuzzy coat on their internal surface, were seen scattered amongst the rod-like profiles (Plates 51 and 52). Again, as in the normal Langerhans cell, the fuzzy coat could be seen to be continuous with that lining the external membranes of the rod-like portions. Occasional granules were seen attached to the plasma membrane. Granular and membranous lysosomal bodies were also commonly seen (Plates 50 - 52).

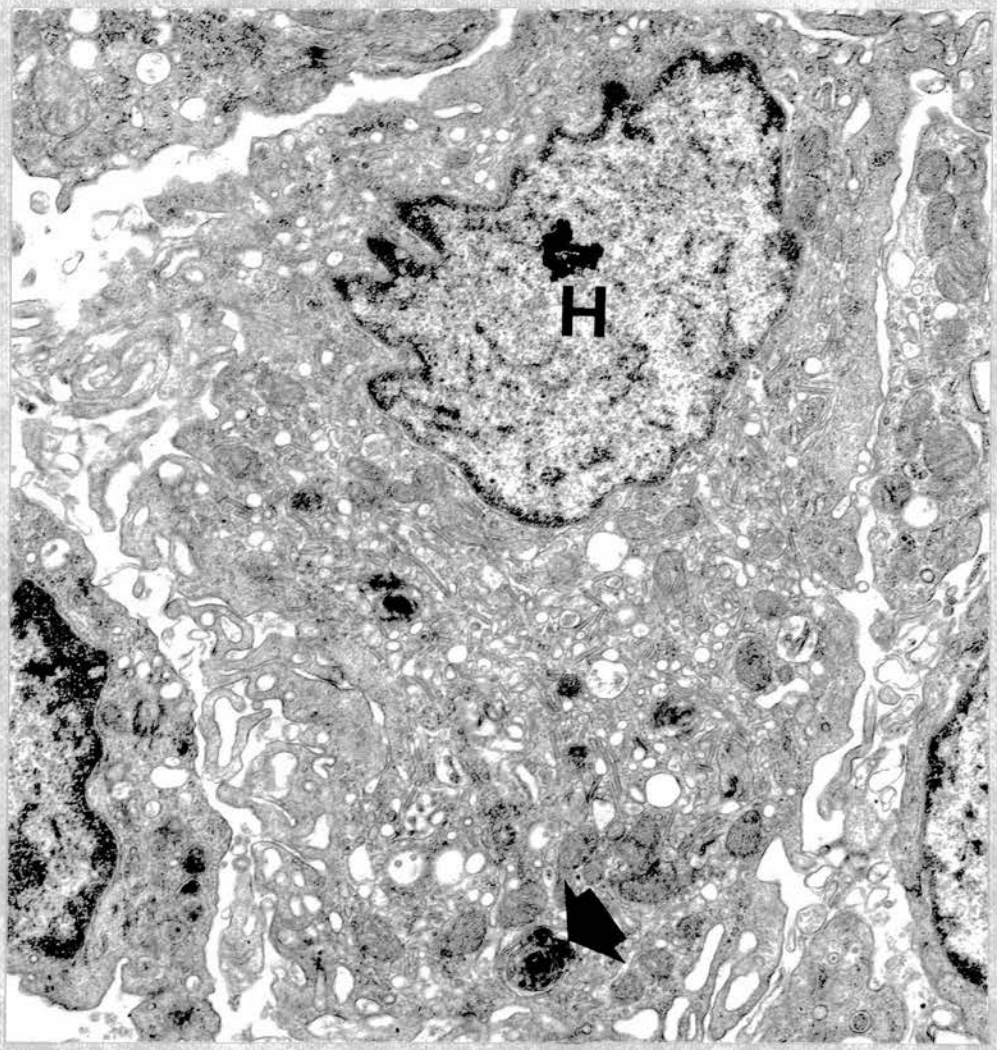


Plate 51 (X 14,500): Histiocytosis X cell (H). Numerous Langerhans cell granules are seen. Arrow points to lysosomal structure.

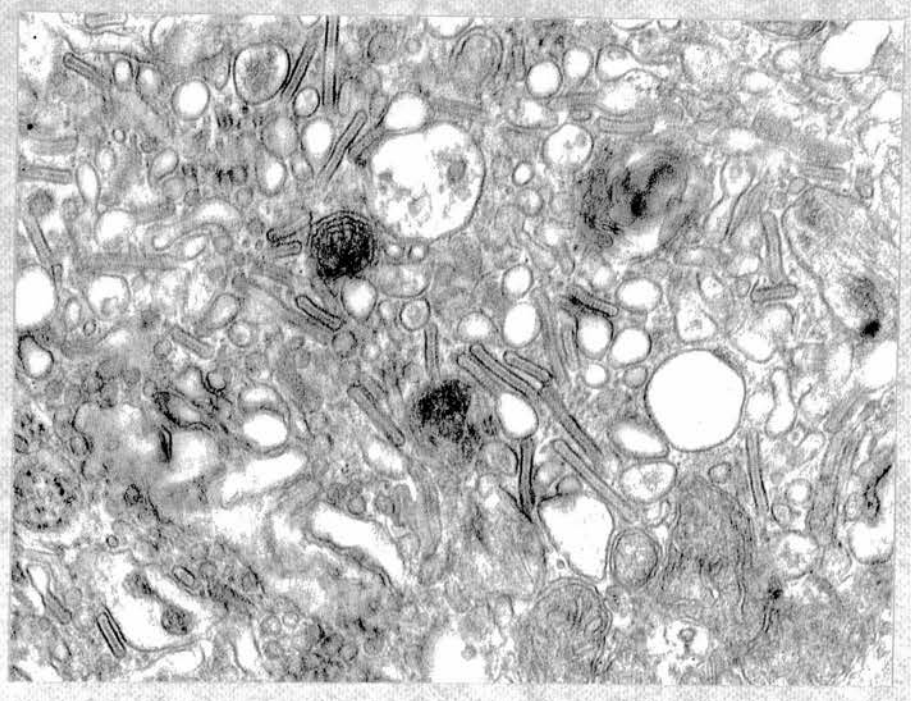


Plate 52 (X 43,000): Histiocytosis X. Part of cell shown in Plate 51.

4. DISCUSSION

The clinical and pathological features of this case are not unusual. The age of onset and the rapid course of the disease are typical, though the skin lesions were rather scanty and the process affected mainly the lungs. Although this is perhaps not a textbook presentation of Letterer Siwe disease it is a well recognised combination of organ involvement by the infiltrate (Clark et al., 1970). In view of the three month history of gum trouble, it was unfortunate that the infant did not come into medical hands earlier, especially as curable forms exist (Nezelof et al., 1973).

The ultrastructure of skin lesions reported here is similar to that noted by numerous other workers (e.g. Turiaf and Basset, 1966; Cancilla et al., 1967; Gianotti et al., 1968; Tusques and Pradal, 1969 and Morales et al., 1969), since the original description of Basset et al. (1965). Tusques and Pradal (1969) carried out careful measurements on three dimensional reconstructions of the granules in histiocytosis X cells and came to the conclusion that there were only minor differences between the granules of histiocytosis X and those of epidermal Langerhans cells. Their measurements are compared with those of others in Table 2.

Auteurs	Objet	Fixation	Distance entre les parois externes	Distance entre les parois internes	Epaisseur du filament axial	Périodicité	Longueur	Epaisseur de la paroi externe
BASSET et coll., 1965	Histiocytose X	OsO_4	420 Å	330 Å	110 Å	120 Å	plus de 1 µ	
BOUISSOU, 1967		OsO_4	500 Å	320 Å	70 Å	120 Å	0,8 µ	
DE MAN, 1968		OsO_4	400 Å		100 Å	130 Å		
TUSQUES et PRADAL, 1968		OsO_4	410 Å	315 Å	90 Å	115 Å	plus de 2 µ	30 Å
BREATNACH, 1965	Cellules de Langerhans	OsO_4	400 Å			90 Å	0,5 µ	150 Å
WOLFF, 1967		OsO_4	500 Å			50-60 Å	plus de 1 µ	55-60 Å
SAGEBIEL et REED		OsO_4				90 Å		

Table 2: Comparison of granule measurements in histiocytosis X and Langerhans cells.
(Paroi, wall; epaisseur, thickness)
(From Tusques and Pradal, 1969)

There is now little disagreement that the granules in some histiocytosis X cells are structurally identical with those of the epidermal Langerhans cell. Wolff (1972) supports this view, and comments that there are only four minor differences between Langerhans cells

and histiocytosis X cells containing the specific granule.

1) In histiocytosis X the granules branch more often and form interconnected networks.
2) More granules are attached to the cell membrane in histiocytosis X.
3) Granules can be found in the nucleus of histiocytosis X (Wolff and Sollereder, 1969), presumably as the result of abnormal mitosis.
4) Granules are abundant in degenerating cells of histiocytosis X (Tusques and Pradal, 1969), probably because they are very resistant to destruction (Nezelof et al., 1973).

1) and 2) only were noted in this study.

The marked membranous activity of the histiocytic cell wall, and the increased number of granules attached to the wall and seen in the periphery of the cell have suggested an endocytic origin of the granule (see Page 42), (Tarnowski and Hashimoto, 1967; Hashimoto, 1971). The fact that similar granules have been noted in monocytic leukaemic cells exposed to agents provoking phagocytosis (thorotrast and pseudomonas aeruginosa) also favours the idea that the granules are formed as a result of a cell surface phenomenon (Sanel and Serpick, 1970).
However Niebauer et al. (1969) felt that the staining properties of the granule with osmium zinc iodide favoured an origin from the Golgi apparatus. Gianotti et al. (1968) considered that the granules were

"rather non specific cytoplasmic organelles" and most probably originated from numerous cell membrane systems.

Histogenetic arguments for a Langerhans cell origin of the cells in histiocytosis X have been presented most persuasively in an excellent review by Nezelof et al. (1973). They suggest that the morphological and cytochemical evidence favours the view that histiocytosis X is due to a localised or disseminated proliferation of pathological Langerhans cells, which exhibit a high phagocytic capability, abundant cytoenzyme equipment, and preserve these characteristics in <u>in vitro</u> culture (Basset and Nezelof, 1969). They point out that the distribution of lesions (bone, skin and lungs in particular) is different from that of other tumours of the haematopoietic system, which emphasizes the individuality of the disease.

The skin, (particularly the scalp and external auditory meatus) and mucous membranes (e.g. those of the gums) are frequently the first regions to be involved in histiocytosis X, and so it is quite possible that there is initial proliferation of Langerhans cells in these sites with subsequent spread via the blood. It is of interest that Langerhans cells have been noted in the blood in this condition (Inamura et al., 1971).

The destructive character of histiocytosis X lesions can be explained by enhanced phagocytic and secretory

functions of Langerhans cells (Nezelof et al., 1973). Epidermal destruction, in particular, may be due to the increased production of a substance toxic to keratinocytes, which normal Langerhans cells might produce (Prunieras, 1969).

Nezelof and Basset (1973) have enumerated the common and contrasting characteristics of histiocytosis X cells and histiocytes.

In common they exhibit:

1) a similar size
2) nuclei with scalloped edges
3) adherence to glass
4) similar enzymatic equipment (see Chapter I)
5) phagocytic activity (e.g. erythrophagocytosis) which can be demonstrated in vivo and in vitro.

They differ in their:

1) survival in vitro (histiocytosis X cells survive longer)
2) developmental characteristics in vitro. (Histiocytosis X cells often form large giant cells in vitro and preserve their morphologic individuality (Basset and Nezelof, 1969) whereas human histiocytes rarely form giant cells, and may transform into fusiform cells (Kouri and Ancheta, 1972).

The relatively mild nature of histiocytosis X, in contrast to the rapidly fatal course of the malignant histiocytoses, (reviewed well by Abele et al., 1972) is

striking. It seems probable that these two groups of histiocytic diseases occupy opposite ends of a spectrum. Histiocytosis X can be considered to be a proliferative disorder of a highly differentiated and specialized cell (the Langerhans cell) with consequent low malignancy, whilst the malignant histiocytoses are due to stem cell anaplasia with high malignancy (Nezelof and Basset, 1973). Transitional forms can be expected to occur and it is interesting to note that Langerhans cell granules were found in the proliferating cells of a case that satisfied the clinicopathological criteria of both Letterer Siwe disease and malignant histiocytosis (Imamura et al., 1971). Henderson and Sage (1973) also found Langerhans cell granules in a case of malignant histiocytosis.

It should perhaps be emphasised that the diffuse histiocytic malignant lymphomas, or reticulum cell sarcomas in older terminology, (see Stuart, 1975 for discussion of terminology) do not usually have a histiocytic origin. Tumour cell surface receptor studies (Habeshaw and Stuart, 1975) on seven cases indicated that the majority were derived from lymphocytes. However a histiocytic origin appeared probable in one case whilst the origins of two tumours was unknown, as the cells bore no identifiable receptors.

Chapter VIII

MALIGNANT MELANOMA

Including

1. INTRODUCTION
   - a) Clinical features
   - b) Histogenetic pattern
   - c) Purpose of this study

2. MATERIAL AND METHODS
   - a) Histogenetic typing
   - b) Electron microscopy

3. RESULTS
   - a) Histogenetic typing
   - b) Electron microscopy
     - i. General cellular characteristics
     - ii. Melanosomal morphology
     - iii. Dopa reaction

4. DISCUSSION
   - a) Findings in this study
   - b) Comparison with other studies
   - c) Pathogenesis of malignant melanoma

1. INTRODUCTION

It has been estimated that there are about 2000 million melanocytes in human skin (Szabo, 1967). Benign tumours derived from them are the commonest of all skin tumours: a mirror rather than a visit to the skin clinic is all that is needed to see naevi and lentigines. Although malignant tumours of melanocytes are rare they continue to attract the attention of both the profession and public. This could be due to the appreciable incidence of the tumour in younger age groups or perhaps its occasional extraordinary biological behaviour. More probably, however, the explanation lies in the common awareness of a potentially tragic sequence of events triggered off by the appearance, or change of a trivial black spot.

a) Clinical features

Only recently has it been appreciated that there are three major types of invasive malignant melanoma (Clark et al., 1969; McGovern, 1970). All differ in their mode of onset, course and prognosis. Each has a distinctive clinical and histological appearance. Two are preceded by an in situ phase where there is intraepidermal proliferation of malignant melanocytes (Fig. 14).

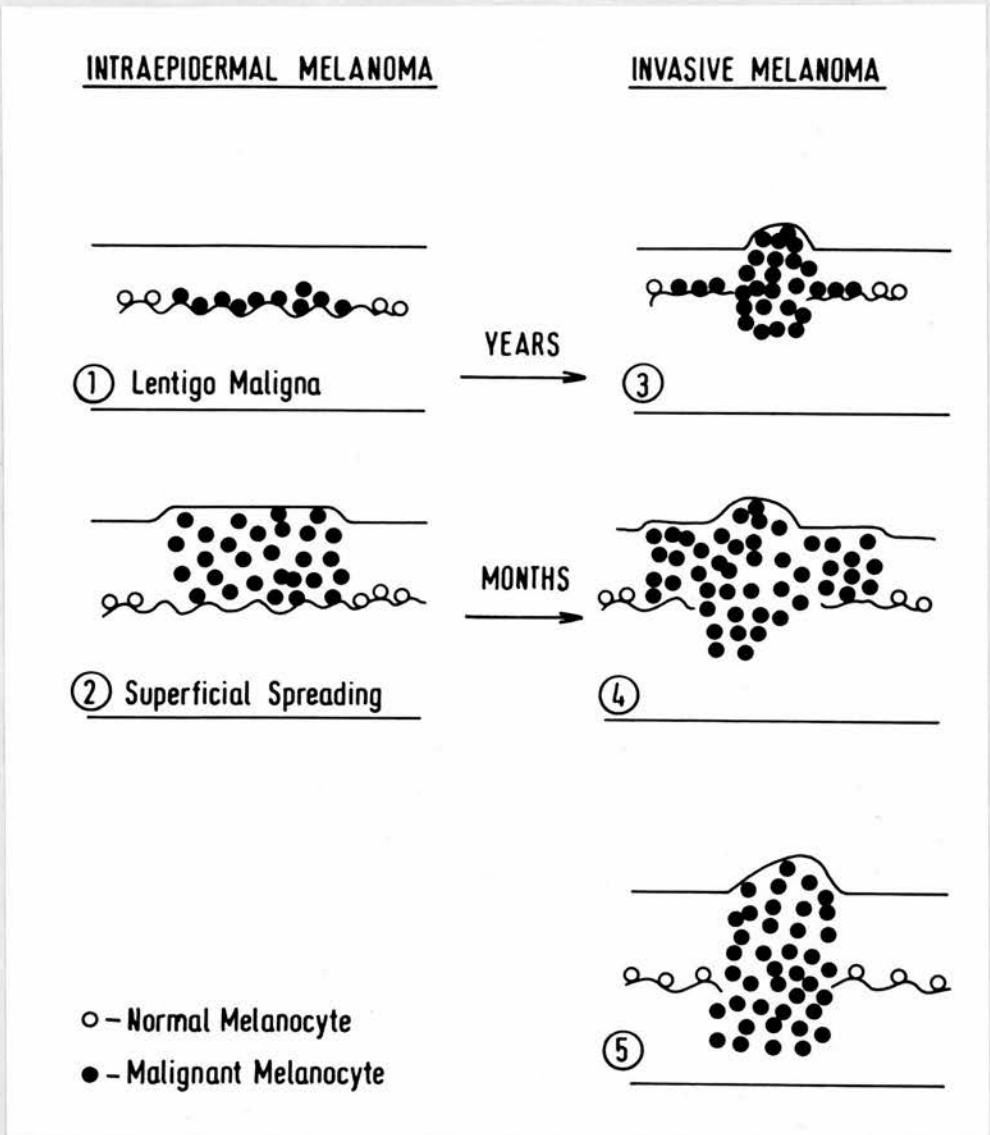


Figure 14: Diagram to show evolution of different histogenetic types of malignant melanoma.

The invasive lentigo maligna melanoma (Fig. 14 - (3)) is preceded by the in situ change of lentigo maligna (Hutchinson's freckle, mélanose circonscrite précancéreuse, Dubreuilh's melanosis circumscripta praecancerose) shown in Fig. 14 as (1). The invasive superficial spreading melanoma (Fig. 14 (4)) is preceded by the superficial spreading melanoma in situ (pagetoid melanoma, premalignant melanosis) shown in Fig. 14 as (2). Invasive nodular melanoma (Fig. 14 - (5)) appears de novo - with no antecedent spreading pigmentation.

Lentigo maligna (Plate 53) is a slowly increasing irregular patch of pigmentation seen mostly on the face of old patients. The lesion is not indurated and consists of all shades of light brown to black (Clark et al.,1969). Spontaneous regression in some areas causes patches of relative depigmentation. The lesion may grow slowly for years before dermal invasion, seen as an indurated or nodular area, occurs. Only about a third become invasive (Lever, 1975) and not usually until they have been present for ten to fifteen years and have reached 4 - 6cms in diameter (McGovern, 1970).

Lentigo maligna melanoma (Plate 53) is therefore seen as a nodule (often bluish black) developing within a patch of lentigo maligna.

Superficial spreading melanoma in situ is seen usually on the non-exposed skin of the middle-aged. It is slightly elevated and therefore palpable. Its outline is often arciform with prominent indentations. The colour variations are similar to those of lentigo maligna except more shades of pink and blue are seen (Clark et al., 1969). In contrast to lentigo maligna, invasion always occurs within one to two years of the appearance of the lesion, usually before it is larger than 2 - 3cms. Again induration, the development of a nodule or ulceration and bleeding are pointers suggesting dermal invasion, i.e. transition to an invasive superficial spreading melanoma (Plate 54).

Nodular malignant melanoma (Plate 55) starts as an elevated, often deeply pigmented, nodule which grows rapidly and frequently ulcerates. Although relatively amelanotic variants occur, careful examination (with a magnifying glass) usually reveals flecks of pigmentation.

This clinical classification can be conveniently used in most cases, though on a few occasions there is some degree of overlap between the three types of invasive melanoma, both clinically and histologically.

It is now thought that only a quarter of malignant melanomas develop from pigmented naevi. About 25% of patients with malignant melanoma recall a pre-existing pigmented lesion present since childhood, and remnants of naevi are seen histologically in only 10% of excised specimens (Clark et al., 1969). Pre-existing pigmented lesions frequently referred to by patients, are probably in situ stages of melanoma rather than naevi. All three clinical types of invasive malignant melanoma may develop from naevi.

Plate 53: Lentigo maligna lateral to angle of mouth. Above this lentigo maligna melanoma has developed in a patch of lentigo maligna.

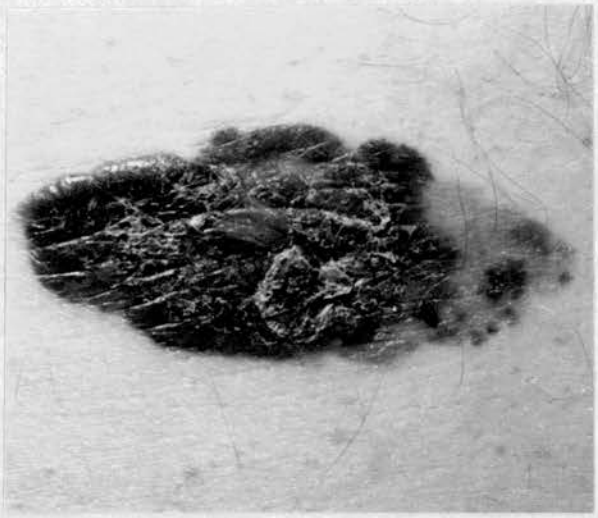


Plate 54: Superficial spreading melanoma on back. Note spontaneous regression on right.

Plate 55: Nodular melanoma. Crusted lesion on back

b) Histogenetic pattern

In 1972 an international group of pathologists met in Sydney, Australia to devise a clinical classification of malignant melanoma and to suggest a more standardised form of histological reporting which would be suitable for international use. Their helpful recommendations were summarised recently (McGovern et al., 1973). The histology of melanoma is also reviewed well by Lever (1975). Lentigo maligna has a characteristic histology (Plate 56). In the very early stages there is hyperpigmentation of the basal cells in the epidermis and the appearance of weird looking elongated and spindle shaped melanocytes throughout the basal layer. The nuclei of these atypical cells show marked pleomorphism. In more advanced lesions the proliferating abnormal cells almost replace the basal cell layer and small clusters are often seen in the preinvasive phase. A helpful clue in diagnosing this histogenetic pattern is the frequent involvement of melanocytes in the external root sheaths of hair follicles. In the superficial dermis adjacent to the epidermal changes there is often a band-like infiltrate consisting of lymphocytes and macrophages filled with pigment. Dermal invasion by the atypical cells signifies transition to lentigo maligna melanoma (Plate 56). There is downward streaming of the tumour cells from the epidermis and the tumour nodule is usually composed of spindle shaped melanoma cells. Pigmentation within the tumour may be minimal but there is often considerable melanin deposition in surrounding melanophages.

Superficial spreading melanoma in situ (Plates 58 and 59). In the preinvasive phase the malignant melanocytes are seen permeating the epidermis in a Pagetoid fashion. From the outset a few similar cells may also be seen in the papillary dermis. Again the atypical cells have a distinctive appearance; they are uniform in shape and size, often contain abundant finely granular cytoplasm and have large nuclei with prominent nucleoli (Plate 59). They are not so pleomorphic as those seen in lentigo maligna. When dermal invasion becomes pronounced the tumour nodule may consist of epithelioid cells, spindle cells, naevus-like cells or combinations of each. When epithelioid cells predominate they frequently form characteristic alveolar formations surrounded by thin fibres of collagen. An inflammatory infiltrate similar to that seen in lentigo maligna is usually present in the neighbouring dermis.

Nodular malignant melanoma (Plate 57)

No preceding intraepidermal phase is seen. In most cases malignant melanocytes at the dermo-epidermal junction invade the dermis and the appearance of the tumour nodule may be indistinguishable from that seen in the invasive form of superficial spreading melanoma. The absence of an intraepidermal component in the adjacent epidermis is an important clue in diagnosing this histogenetic pattern (Plate 57). Occasionally a little intraepidermal invasion may be seen at the side of the tumour but this should not extend more than three rete ridges beyond the edge of the nodule (McGovern et al., (1973).

Plate 56 (X 75): Lentigo maligna melanoma (right) developing in a patch of lentigo maligna (*). (H and E)

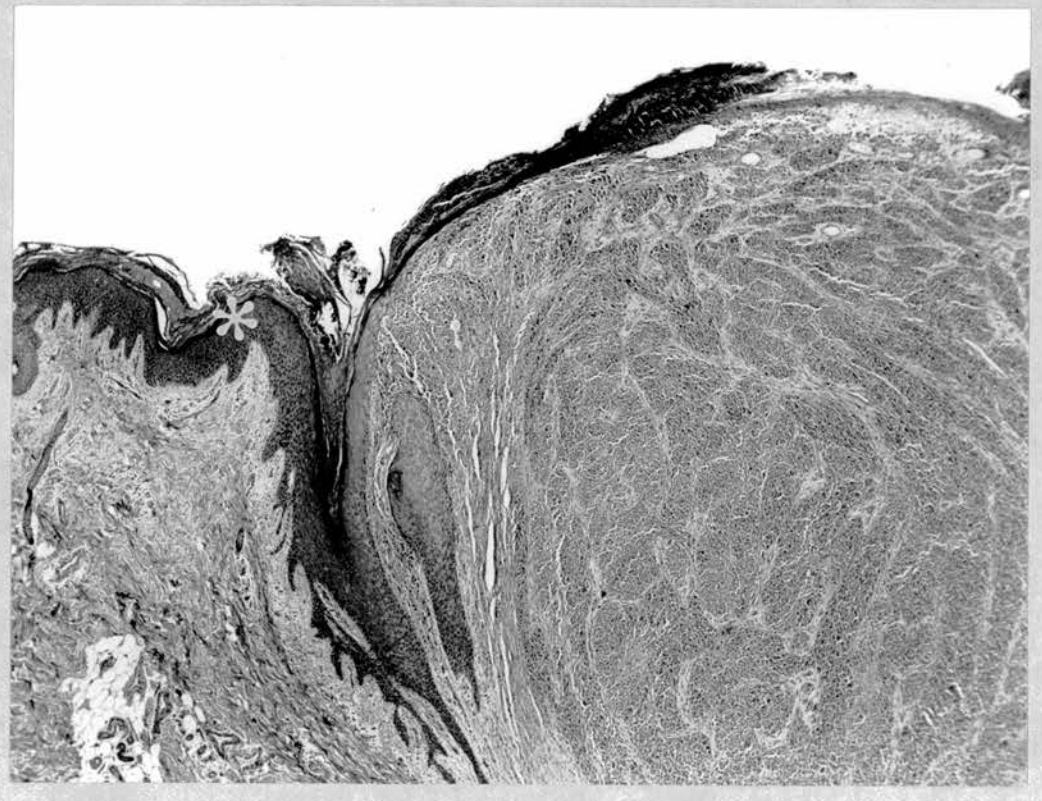


Plate 57 (X 35): Nodular melanoma (right). Note no in situ change in adjacent epidermis (*) (H and E)

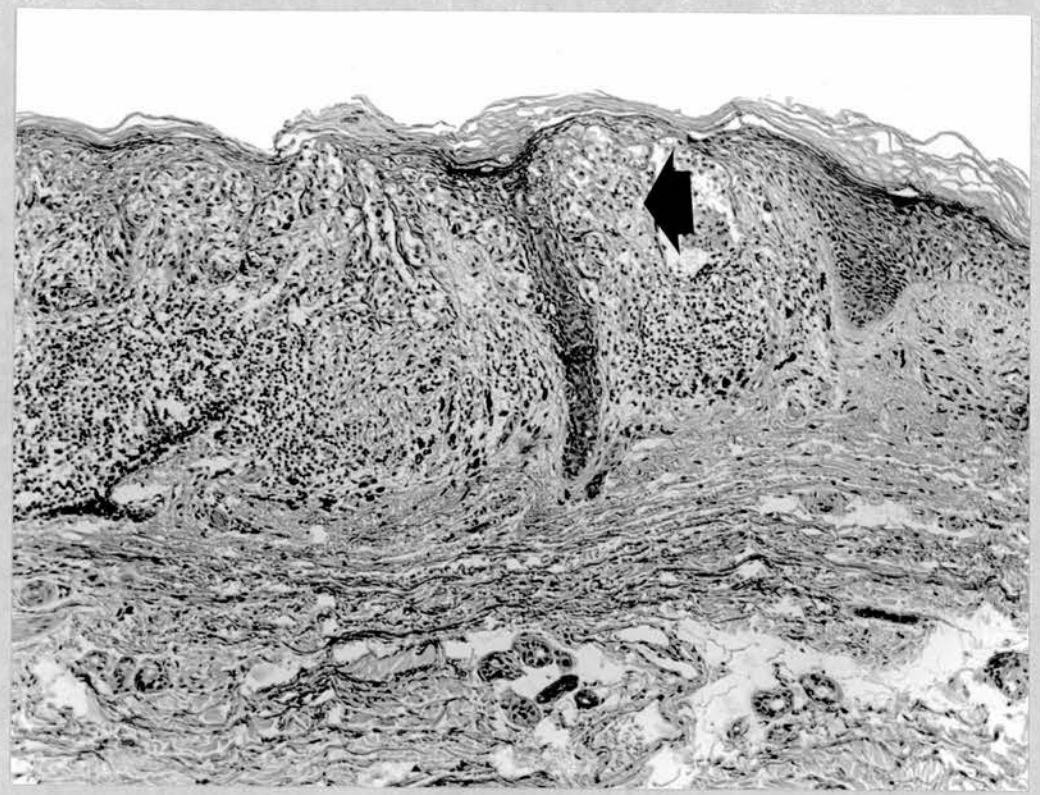


Plate 58 (X 95): Superficial spreading melanoma. The epidermis is permeated with groups of Pagetoid cells and there is invasion into the papillary dermis (arrow). (H and E)

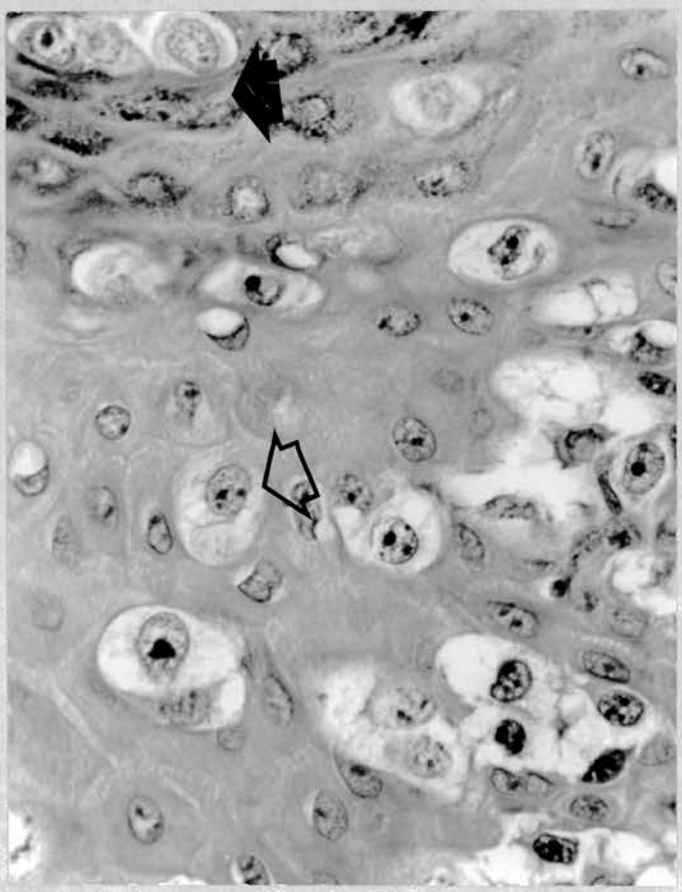


Plate 59 (X 540): Superficial spreading melanoma. Malignant cells (open arrow) permeating epidermis and extending up to the granular layer (arrow). (H and E)

c) Purpose of this study

Although there is general agreement on the different histogenic types of malignant melanoma, it is still not clear whether any of these variations show specific or distinguishing ultrastructural features.

Mishima (1967) should be given credit for being the first to realise that the clinical, histological and ultrastructural features of melanomas arising in a patch of lentigo maligna differed from those of melanomas developing from a junctional naevus. Unfortunately most of his subsequent papers were a catalogue of confusion and, rather than clarify the matter, threw doubt on his original observations and conclusions.

Recently there have been a number of publications on the ultrastructure of malignant melanoma (see Discussion in this Chapter) but in general there has been little agreement between the findings of various groups. Much of the published material can be criticised on two grounds. First of all many studies were concerned with a pitifully small number of patients; they demonstrate the danger of drawing false conclusions from evidence based on inadequate sampling. Secondly, observer bias must have been considerable as all electron microscopy was carried out with prior knowledge of the histogenetic type of the tumour.

The purpose of this study was to determine whether

any, or all, of the three histogenetic types of invasive malignant melanoma have a specific or characteristic ultrastructure. I felt that the best way of investigating this would be to carry out a "blind" study. One group of workers would classify specimens of malignant melanoma according to their histogenetic category on clinical and histological grounds. Other workers, unaware of the histogenetic type, would carry out the electron microscopy and establish the ultrastructure of tumours. After both groups had committed their opinions to print, the code would be broken and the fine structure related to the histogenetic type. It was necessary to enlist the help of others to carry out this project, though my personal role was, by necessity, the major one.

2. MATERIAL AND METHODS

a) Histogenetic typing

Forty surgically excised specimens of malignant melanoma were studied.

Case summaries, clinical photographs and light histological material (representative cuts stained with haematoxylin and eosin and Masson's trichrome stain) were sent to Glasgow (Dr. R. Mackie, University Department of Dermatology and Dr. A.J. Cochran, University Department of Pathology) and the histogenetic type of each tumour determined along the lines outlined

by McGovern et al. (1973).

At the same time the depth of invasion of the primary tumour was established. The levels suggested by McGovern et al. (1973) were used.

Level 1 Tumour confined to the epidermis (intra-epidermal level);

Level 2 Tumour invading the papillary layer but not extending to the reticular layer (papillary-dermis level);

Level 3 Tumour filling and expanding the papillary layer and impinging upon the interface between the papillary and reticular layers but not invading the reticular layer (papillary-reticular-interface level);

Level 4 Tumour penetrating into the reticular layer of the dermis (reticular-dermis level);

Level 5 Tumour invading the hypoderm (sub-cutaneous fat level).

The existence of a preceding naevus was also recorded. This was based entirely on historical evidence and three descriptions only were accepted as indicating a probable preceding naevus: e.g. a pigmented lesion "present all life", "present since a child" or "present as long as can remember". Excluded were pre-existing pigmented lesions which could be traced back for as long as twenty years. It was felt that some true pre-existing naevi might be missed using this method of assessment, but those accepted seemed reasonably certain instances.

b) Electron microscopy

Electron microscopy of tissue obtained from the invasive nodule was carried out in the University Department of Dermatology, Edinburgh (Dr. J. Hunter with help from Dr. S. Zaynoun). At least two (and frequently more) blocks from each tumour nodule were studied, and a minimum of 30 random photographs taken, usually at a machine magnification of X 6,000. The electron micrographs were then analysed independently in Edinburgh (Dr. J. Hunter) and Sheffield (Dr. S. Bleehan, Rupert Hallam Department of Dermatology) both workers still being unaware of the tumour type. For the purposes of this a proforma was designed so that views on particular aspects could be compared (Table 3).

MELANOMA STUDY NO: ☐

(Circle relevant finding) +++ = Nearly all
++ = Frequent
+ = Occasional but rare
- = None

TUMOUR CELLS

Nucleus:	Normal	Convoluted
Nucleolus:	Normal	Abnormal - Compact - Dispersed
Golgi:	Normal	Prominent
Mitochondria:	Normal	Prominent
R.E.R.:	Normal	Prominent
Free Ribosomes:	Normal	Prominent

MELANOSOMES

Size:	nm	nm
Shape:	Ellipsoidal	Spheroidal
	+++ ++ + -	+++ ++ + -
Character:	Normal:	+++ ++ + -
	Abortive:	+++ ++ + -
	Vacuolar:	+++ ++ + -
	Granular:	+++ ++ + -
	Lamellar:	+++ ++ + -
Amount of pigment:	Normal	Much Little
Distribution of pigment:	Normal	Abnormal
Autophagosomal profiles:	None	Few Many

DIFFERENTIATION OF TUMOUR CELLS

Normal (A) Moderate (B) Poor (C) None (D)

CLARK TYPING I II III IV

Table 3: Melanoma Proforma

It can be seen from the proforma (Table 3) that particular attention was paid to melanosome morphology. These were categorised as ellipsoidal or spheroidal and were further subdivided into normal, abortive, granular and lamellar types according to the terminology suggested by Clark et al. (1972). Another type of melanosome, termed vacuolar (see below), was considered because of its frequent occurrence.

Normal melanosomes: The appearance of the melanosomes are as described and illustrated in Chapter III, although they may be larger or smaller than normal. They are ellipsoidal and their internal fine structure is essentially normal, showing individual melanofilaments with distinctive periodicity (100A°) and a tendency to form cross striations.

Abortive melanosomes: Spheroidal organelles containing one or more filaments having the distinctive periodicity of the filaments of a normal melanosome (melanofilament). The filaments are not present in large numbers in parallel array, and show no tendency to form "cross-linkages". Striated appearances are therefore rare in this type of melanosome.

Granular melanosomes: Spheroidal organelles, limited by a unit membrane, and containing an evenly dispersed small granular component.

Lamellar melanosomes: Spheroidal organelles characterised by an internal array of membrane structures of unit membrane form which may form either a concentric whorl or parallel patterns. These melanosomes may also contain clusters of vesicles giving them the appearance of a compound lysosome.

Vacuolar melanosomes: Spheroidal organelles, limited by a unit membrane, but empty. Their melanosomal nature could be identified, only with certainty, by demonstration of their dopa positivity (see below).

Vacuolar melanosomes never contain melanin but pigment deposition may occur in all other types of melanosomes. It may look regular and organised, or totally haphazard in its distribution.

A semi quantitative score system (see Table 3) was used to describe the frequency of each type of melanosome.

Taking into consideration all the fine structural features the observers (Dr. J. Hunter and Dr. S. Bleehan) attempted to grade the degree of cellular and subcellular differentiation within the tumour. (If the tumour cell characteristics resembled normal melanocytes, then this was called 'normal differentiation' (A). 'No differentiation' (D) was the term used to describe tumour cells in which there was no evidence of melanogenesis).

The categories chosen had to be broad in view of the subjective nature of this opinion (see Table 3).

Finally an attempt was made to classify the tumour according to its main type of cell, again using terminology based on that suggested by Clark et al. (1972).

Type I cell: Melanoma cell containing mostly normal melanosomes.

Type II cell: Melanoma cell containing mainly abortive melanosomes.

Type III cell: Melanoma cell containing mainly vacuolar, granular and lamellar melanosomes.

Type IV cell: An undifferentiated cell often containing very few or no melanosomes. The nucleus is frequently convoluted and the cytoplasm crowded with mitochondria and free ribosomes.

Electron microscopic dopa reaction

This was carried out on ten specimens using 0.1% L-3, 4-dopa as substrate. Control experiments, incubating slices in phosphate buffer alone, were performed in parallel. Methodological details are as outlined on page 91 though in these experiments thin slices of tissue were obtained using a Smith and Farquhar tissue sectioner (I. Sorvall, Inc.) according to the maker's instructions.

3. RESULTS

a) Histogenetic typing

Study of case summaries, clinical photographs and light histological material revealed that the series included:

Lentigo maligna melanoma	14
Superficial spreading melanoma	14
Nodular melanoma	9
Secondary cutaneous deposit	3

Table 4: Series of 40 malignant melanomas classified according to histogenetic type

Representative clinical appearances are seen in Plates 53, 54 and 55 and typical histological features are illustrated in Plates 56 - 58.

Depth of invasion

Using the levels suggested by McGovern et al. (1973) (see page 177) the figures were:

	Level II	Level III	Level IV	Level V
Lentigo maligna melanoma	4	4	4	2
Superficial spreading melanoma*	2	5	3	3
Nodular melanoma	0	2	5	2

* Level not classifiable in one specimen

Table 5: Number of patients categorised according to tumour type and depth of invasion.

b) Electron microscopy

(i) General cellular characteristics

There were only minor variations in the general cellular architecture of tumours of different histogenetic type. The nuclei in tumour cells of all types were frequently bizarre and convoluted (Plates 60 - 62). The nucleoli were often abnormally prominent and the nucleolonema appeared either as a compact mass (Plate 61) or as dispersed strands (Plate 62). Mitochondria and free ribosomes were plentiful in numerous tumour cells and the Golgi apparatus was prominent in some. However no particular picture was characteristic of any histogenetic type.

Tumour cells in lentigo maligna melanoma, however, often exhibited numerous dendritic processes; a feature seldom seen in the other types.

In general most cells in a given tumour were similar, and in only one specimen was there much difference between the two blocks examined. However it was obvious that in all tumours there were odd cells which were different from the main type. For instance most cells of a tumour might contain numerous granular melanosomes and a prominent Golgi, but amongst these could be scattered occasional cells with none or very few melanosomes, but numerous mitochondria and free ribosomes. It was therefore important to pick out the main cell type.

Some tumour cells contained obvious homogenous lipid inclusions whereas others contained vacuoles lined by a layer of lipid. Occasionally large (0.8 - 1.4μm) empty vacuoles, with no limiting membrane, were seen (Plate 73) and it seemed likely that they had formed as a result of lipid extraction during the processing procedure. These vacuoles were noted in all histogenetic types with equal frequency and were quite different from vacuolar melanosomes, which were smaller (150 - 400nm) and lined by a unit membrane.

Langerhans cells appeared normal in the epidermis overlying the tumour nodules, though on a few occasions they contained phagocytosed melanin (Plate 76).

Macrophages containing pigment (melanophages) (Plate 75) and other mononuclear inflammatory cells were seen amongst the tumour cells in all specimens. They were not studied in detail, but their presence or absence did not seem typical of any histogenetic type. Sometimes it was not easy to differentiate between melanophages, and tumour cells showing marked autophagocytosis of melanin. However, in the latter, some melanosomes disposed individually (rather than in complexes) were apparent (Plate 74).

(ii) Melanosomal morphology

Particular attention was paid to the type of melanosome seen in the tumour cells. Plates 63 - 71 illustrate the characteristic appearance of each type. Once again no particular profile was unique in a single tumour, but it was usually easy to determine the most common forms in each tumour, and their occurrences were noted on the proforma.

The degree of autophagocytosis of melanosomes (Plate 74) within tumour cells was also recorded on the proforma.

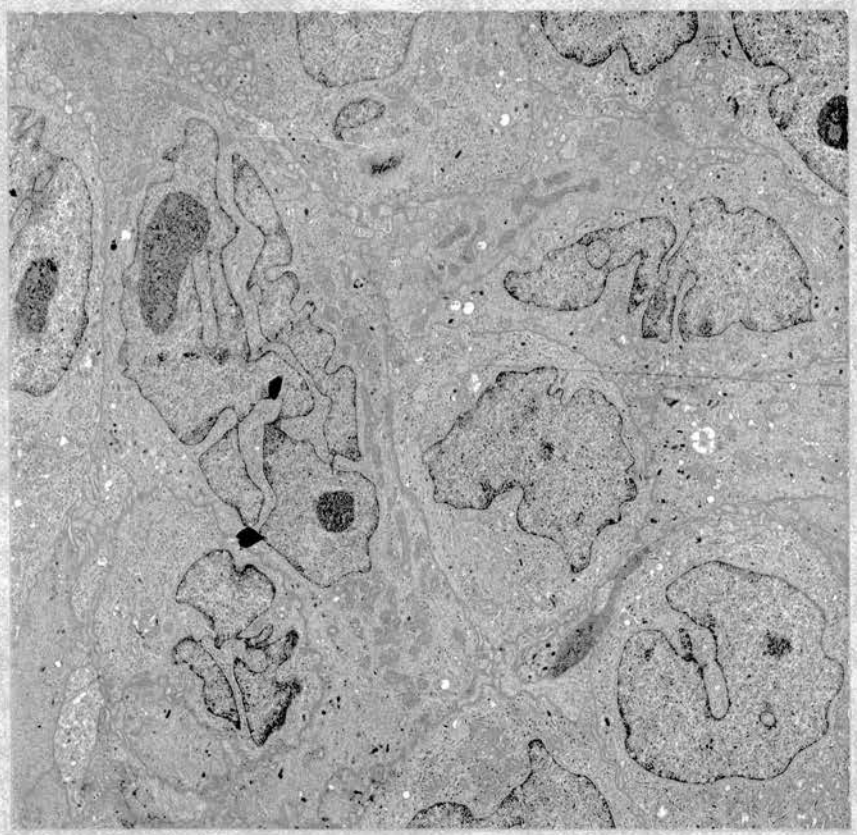


Plate 60 (X 4,000): Malignant melanoma. Convoluted nuclei and prominent nucleoli in tumour cells.

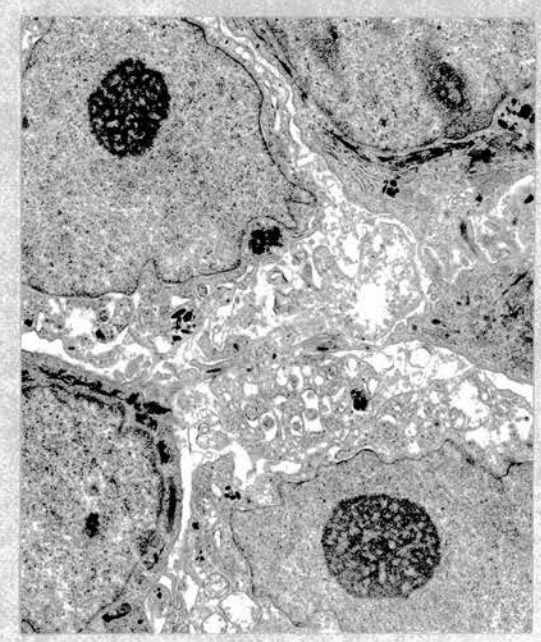


Plate 61 (X 6,000):
Malignant melanoma
Prominent and compact nucleoli in tumour cells.

Plate 62 (X 16,500)
Malignant melanoma
Nucleolus composed of concentric strands of nucleolonema.

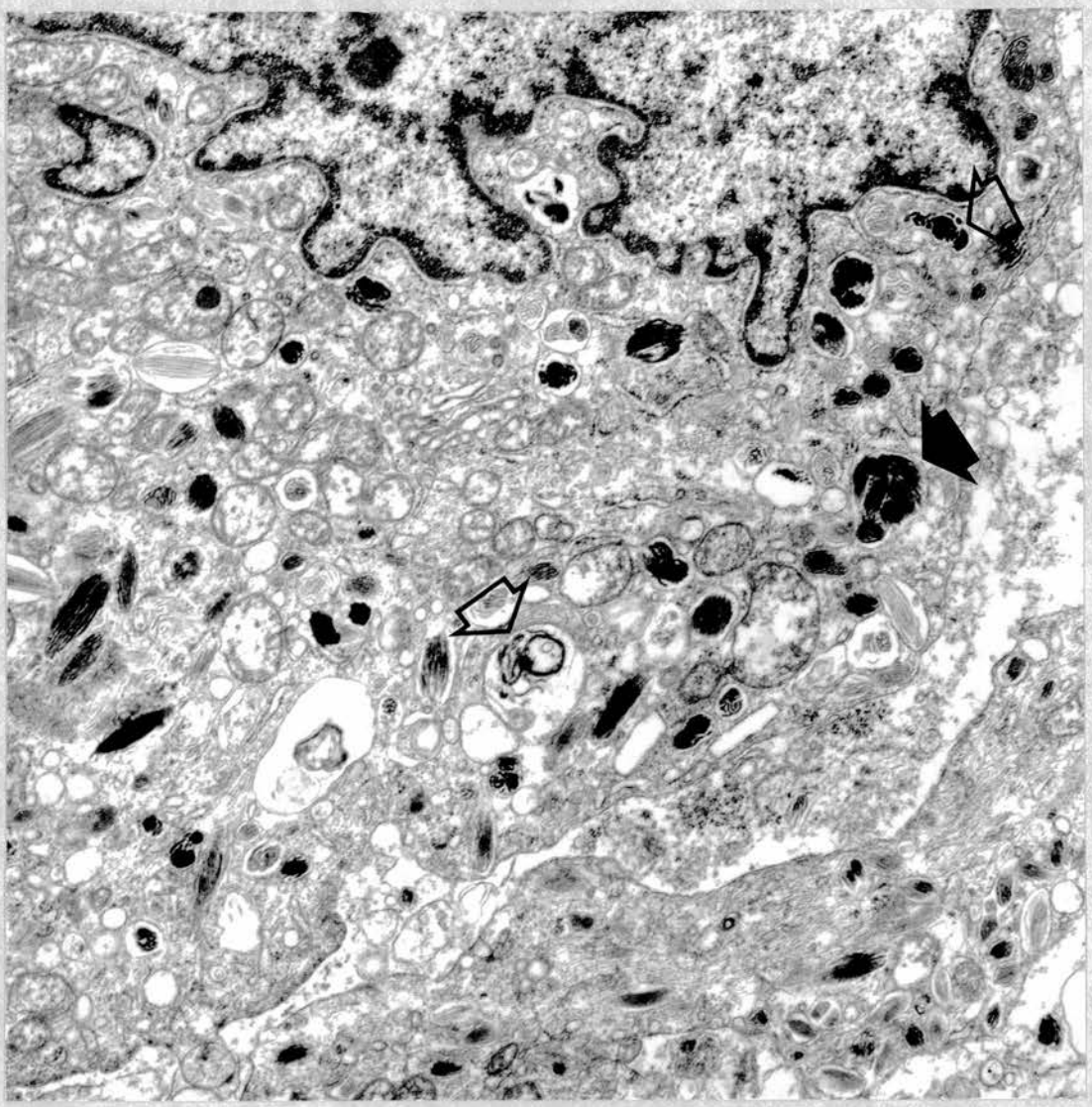


Plate 63 (X 16,000): Type I cell with numerous relatively normal ellipsoidal melanosomes. Occasional abnormal melanosomes (open arrows) are seen. Arrow points to autophagic vacuole.

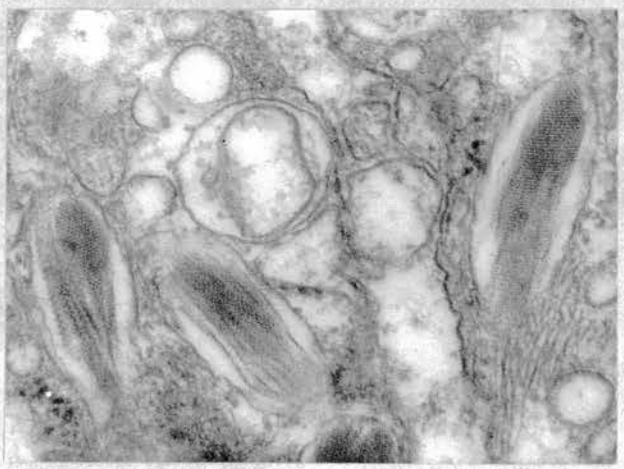


Plate 64 (X 57,000): Ellipsoidal melanosomes in Type I cell Filaments show cross striation (periodicity = 80A°).

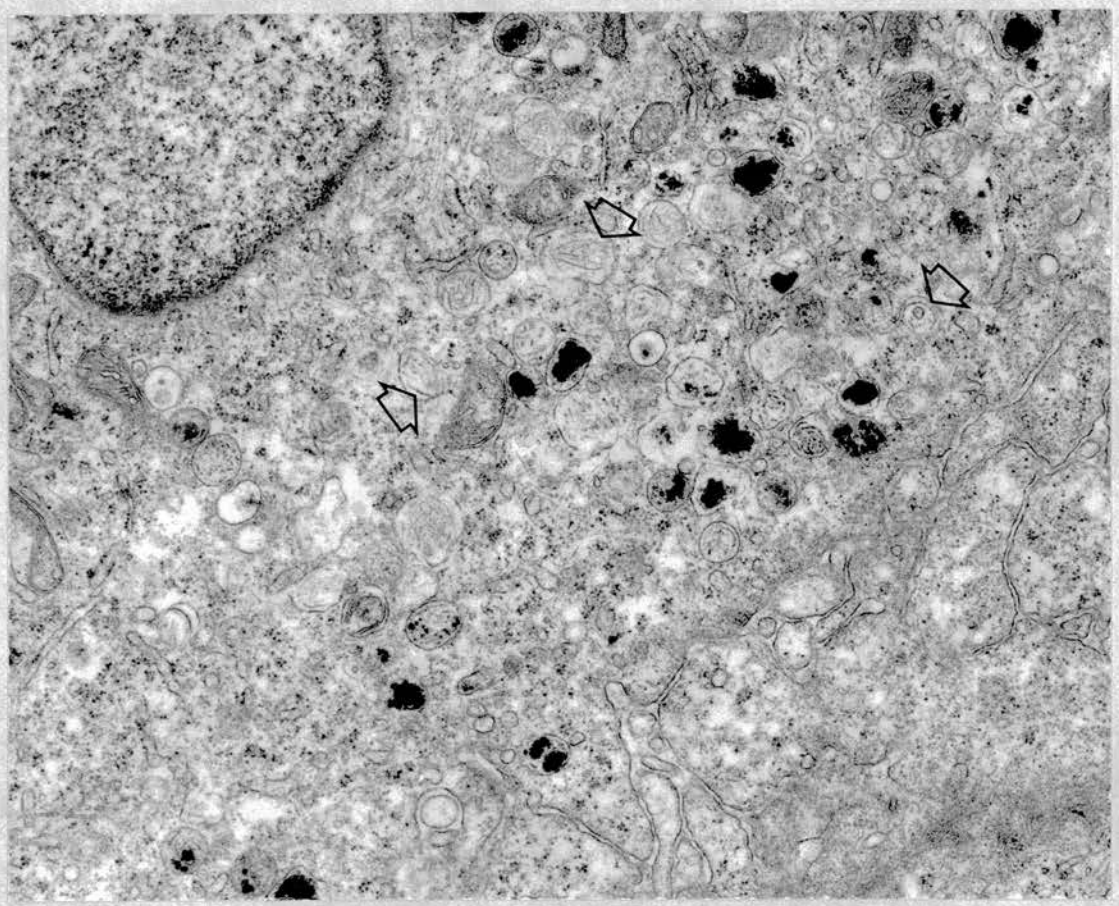


Plate 65 (X 21,000): Type II cell containing numerous abortive melanosomes (open arrows). Filaments in these are orientated in a haphazard manner and there is no cross linking. Irregular pigment deposition is seen in many melanosomes.

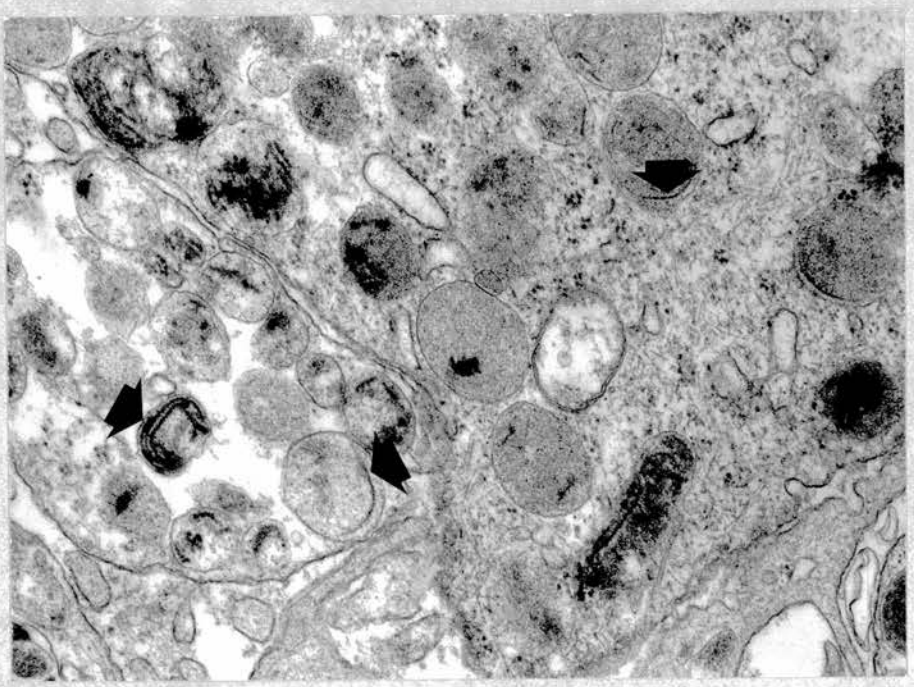


Plate 66 (X 32,000): Abortive melanosomes (arrowed)

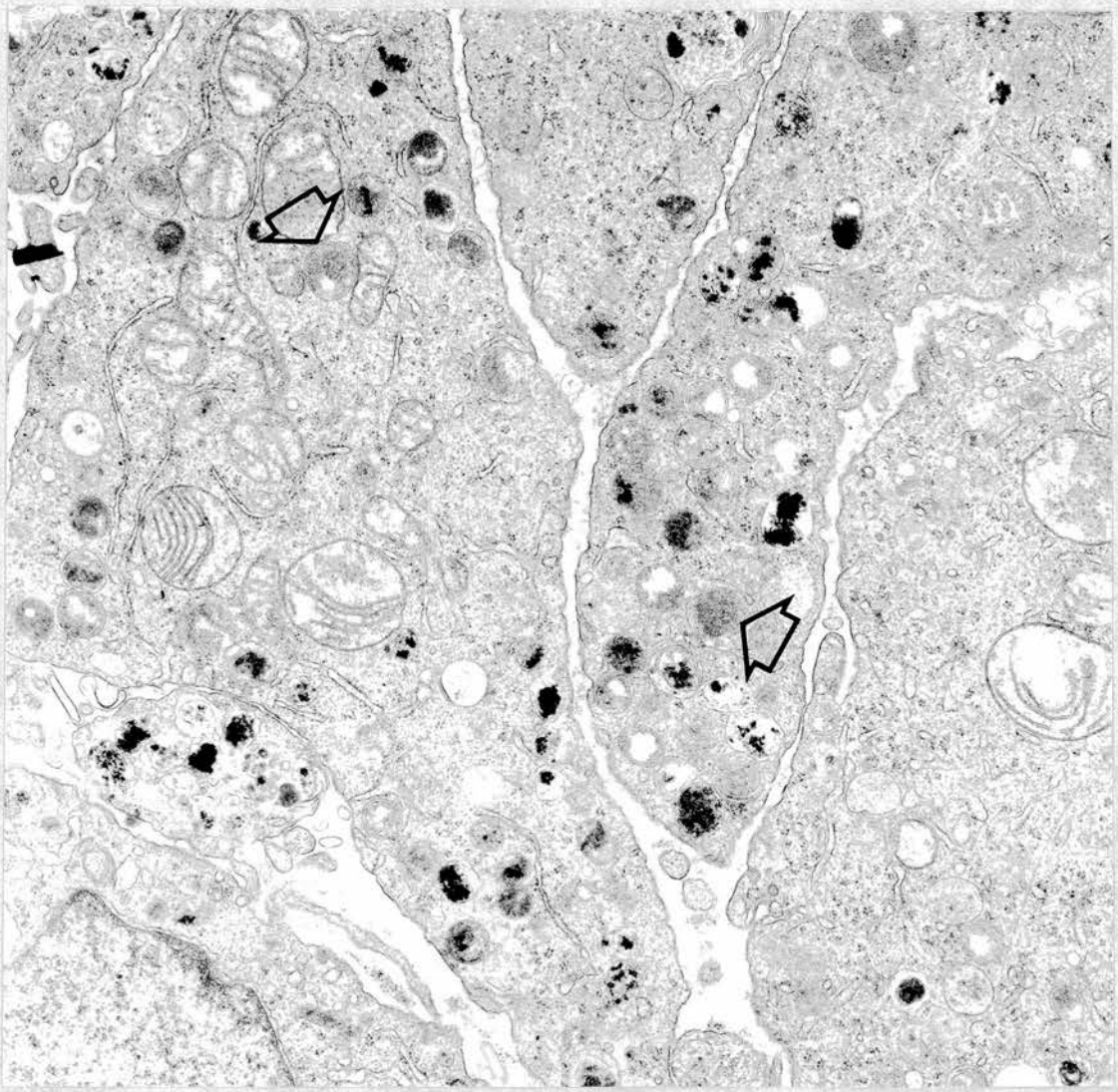


Plate 67 (X 16,000): Type III cell containing numerous granular melanosomes (arrows) as well as occasional abortive and lamellar organelles. Random pigment deposition is seen in many melanosomes

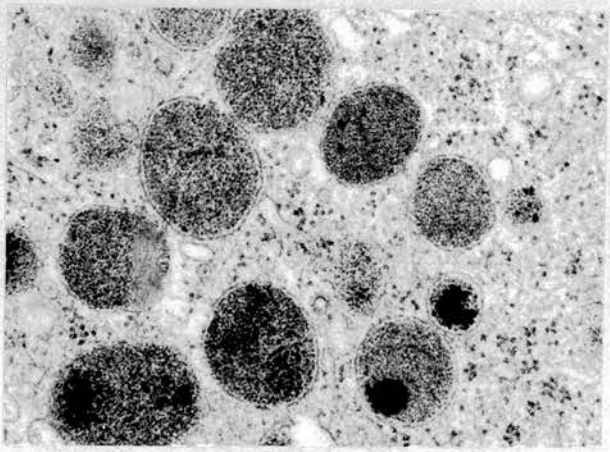


Plate 68 (X 37,000): Granular melanosomes. There is pigment deposition in some.

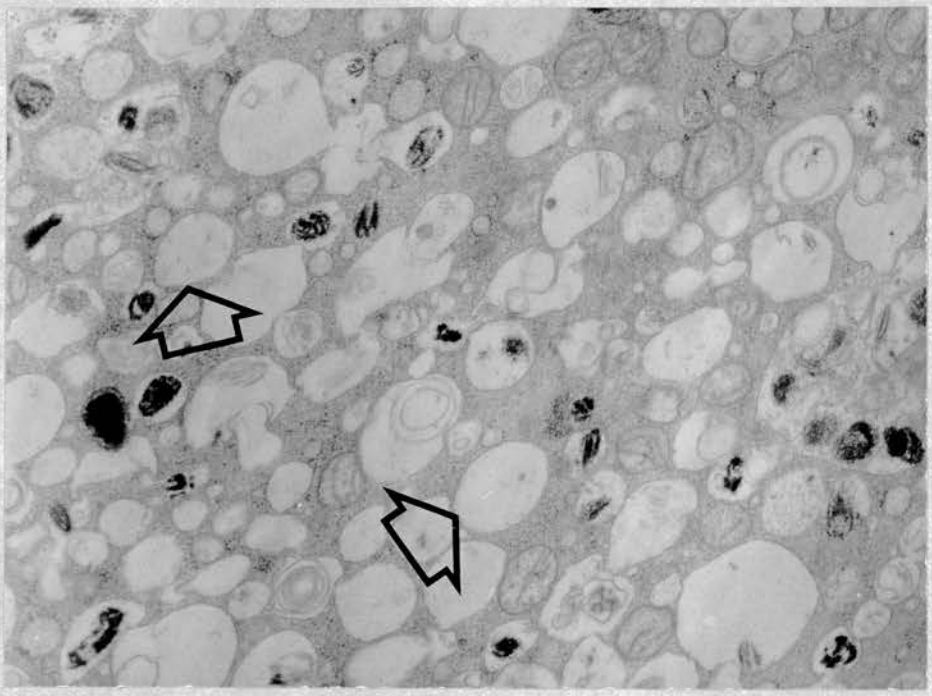


Plate 69 (X 19,000): Vacuolar melanosomes (arrowed). Empty vacuoles (300 - 600nm in diameter) lined by a limiting membrane surrounded by other abnormal spheroidal melanosomes.

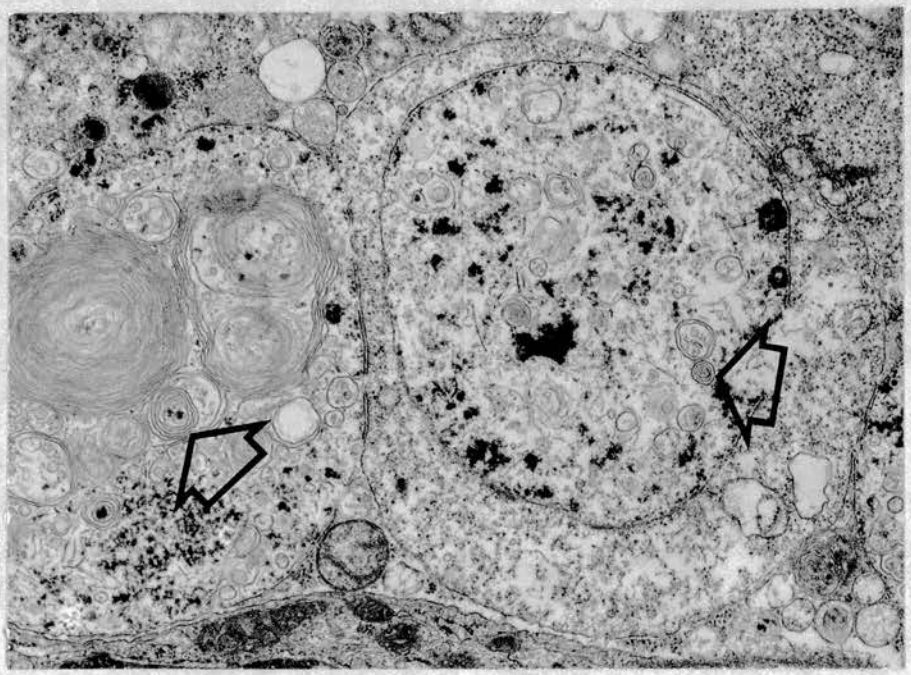


Plate 70 (X 18,500): Lamellar melanosomes (arrowed). The organelle arrowed on the right lies within a large autophagic vacuole.

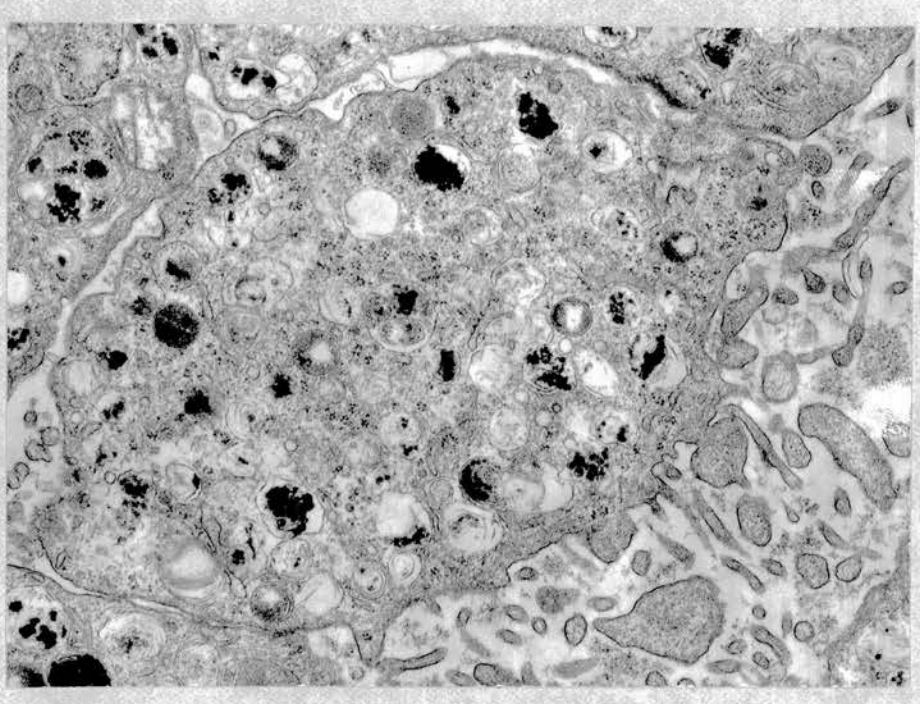


Plate 71 (X 18,000): Type III cell with abortive, granular and lamellar melanosomes.

Plate 72 (X 11,500): Type IV cells. Free ribosomes are prominent, but very few melanosomal structures are seen.

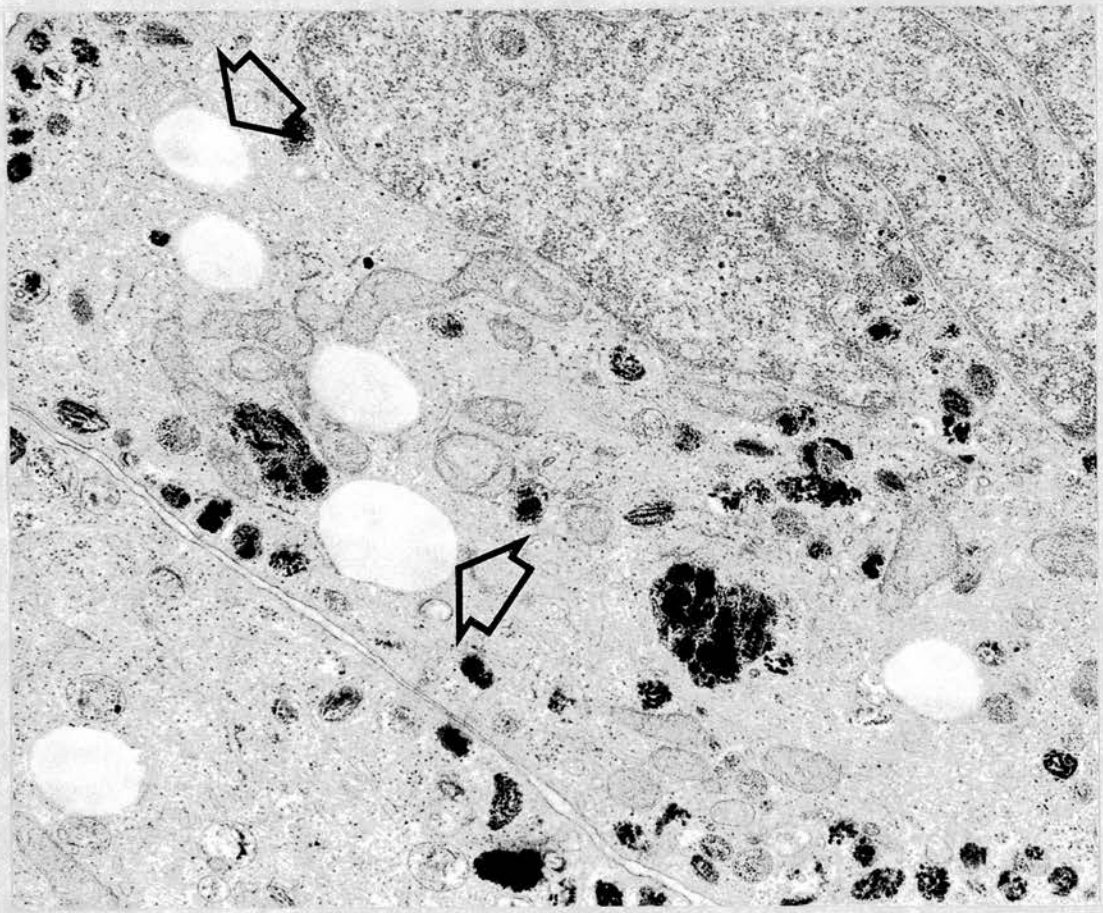


Plate 73 (X 24,000): Vacuoles (up to 0.8μm in diameter) in tumour cells.

There is no limiting membrane to the vacuoles, which have probably developed as a result of lipid extraction in the processing procedure.

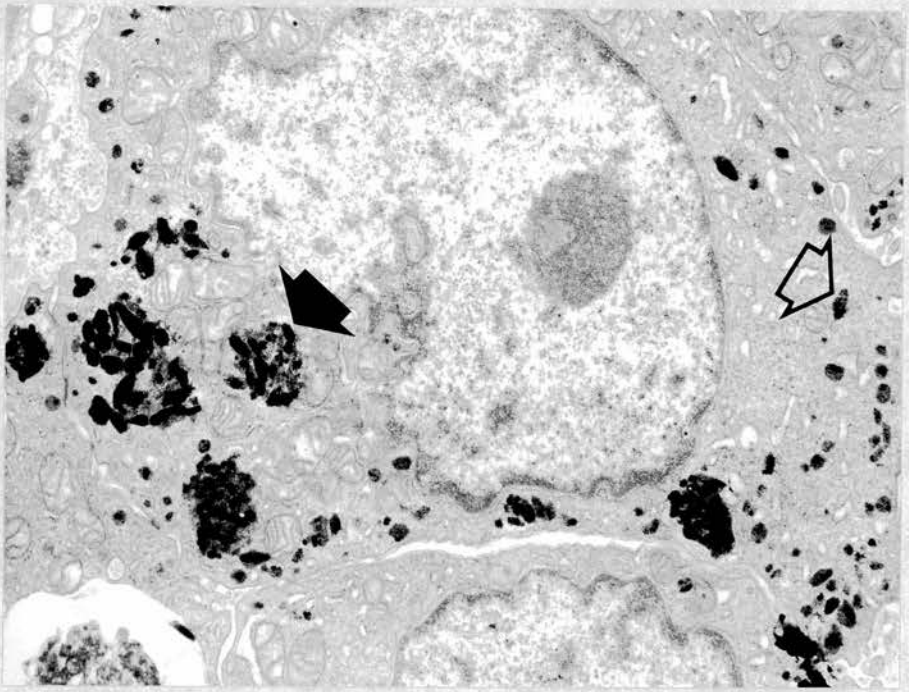


Plate 74 (X 10,000): Autophagocytosis in tumour cell (arrow). Note melanosomes disposed individually (open arrow) in same cell.

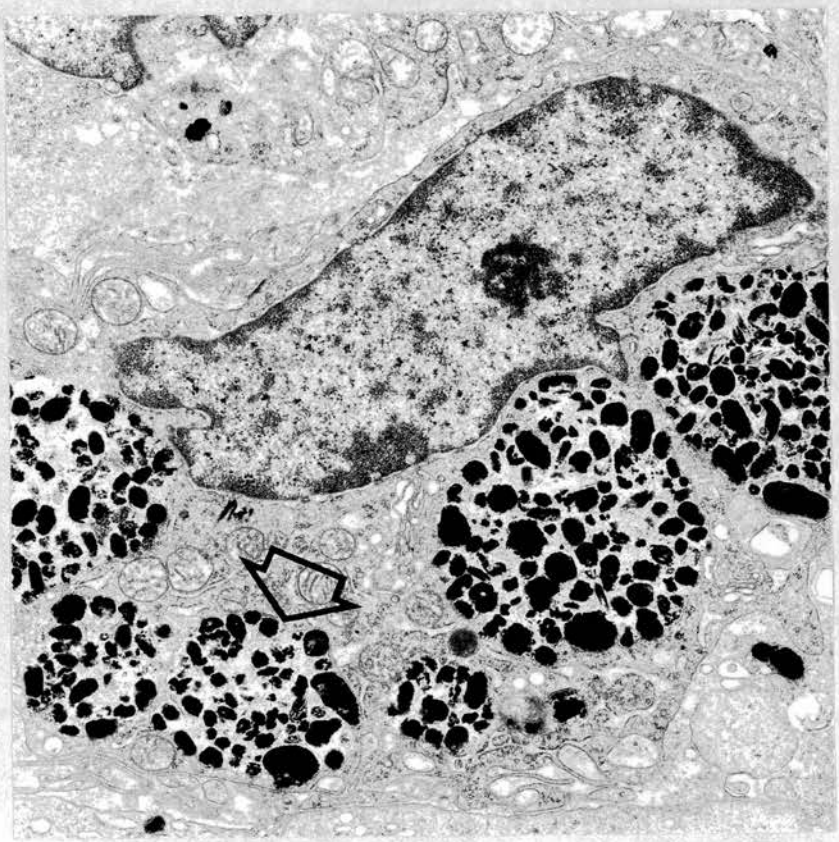


Plate 75 (X 11,000): Melanophage amongst tumour cells. Note numerous melanosome complexes (open arrow) but no individually disposed melanosomes in the same cell.

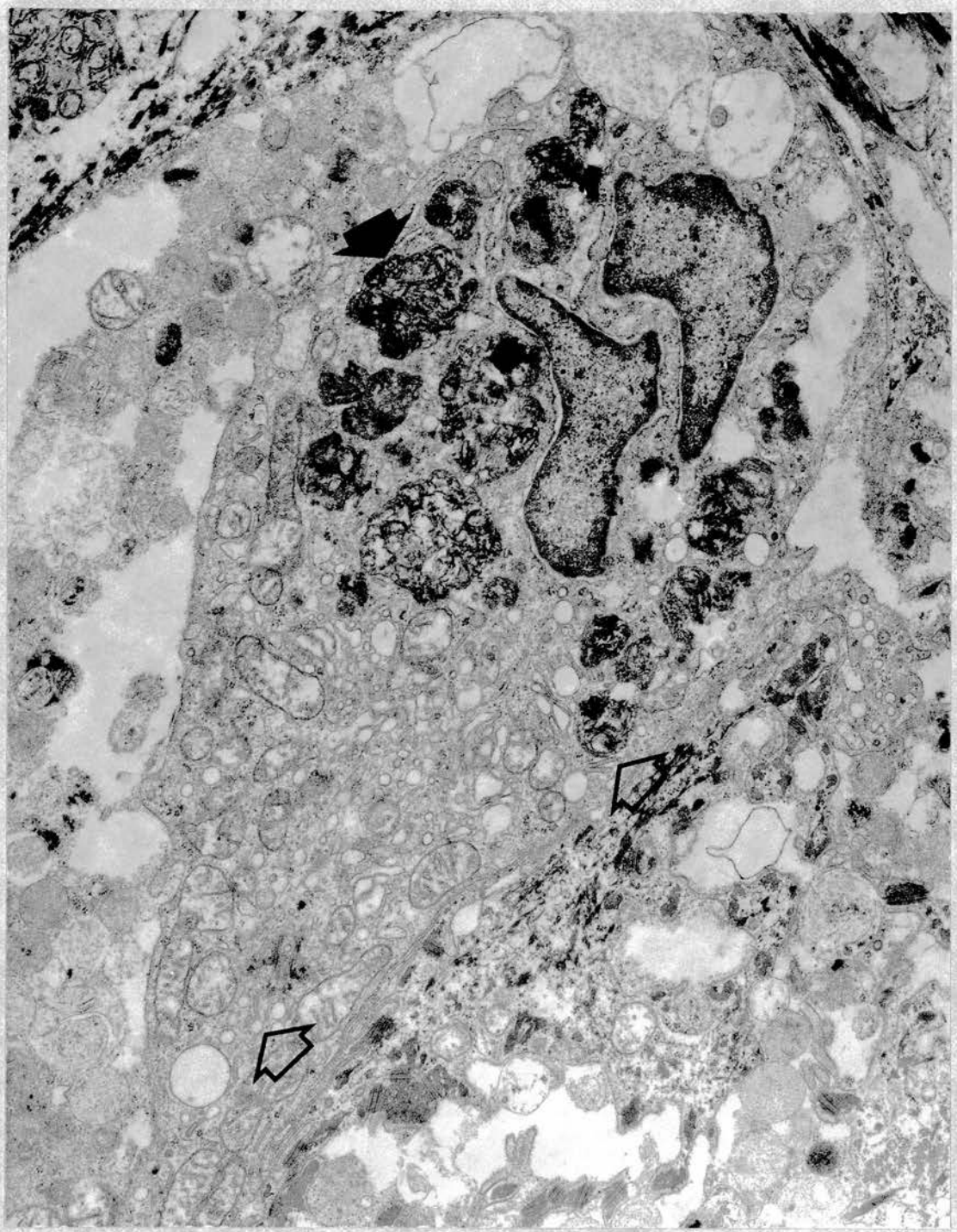


Plate 76 (X 17,000): Langerhans cell in epidermis overlying tumour nodule. There are numerous phagocytic vacuoles containing melanin (arrow). Langerhans cell granules, open arrows.

Eighty proformas were analysed (Dr. J. Hunter and Dr. S. Bleehan each filling up a proforma for every specimen) and there was, in all but a few instances, good agreement between the two workers. Tables 6, 7, 8 and 9 summarise the findings in all the specimens examined.

The ultrastructural appearance of lentigo maligna melanoma is, in the majority of cases, quite characteristic. The cells are frequently dendritic and it is the only type of melanoma in which large numbers of ellipsoidal and normal looking melanosomes (150 - 300nm X 400 - 500nm) are often seen. Granular melanosomes are seen only rarely. In general the cells in lentigo maligna melanoma exhibit a high degree of differentiation, which approaches the picture seen in the normal melanocyte.

The tumour cells in all other types of melanoma are similar. They usually contain numerous spheroidal melanosomes (150 - 500nm in diameter) with only occasional ellipsoidal profiles. Abortive and granular melanosomes are common in all, though lamellar organelles are seen less frequently. Vacuolar melanosomes appear to be more common in superficial spreading melanomas. The tumour cells in superficial spreading, nodular and secondary melanomas are

therefore rather poorly differentiated, and often bear little resemblance to normal melanocytes.

Autophagocytosis is seen frequently in lentigo maligna melanoma and superficial spreading melanoma, but occurs less in nodular melanomas.

Melanomas arising from apparent pre-existing naevi were not characterised by the dominance of any particular type of melanosome. They exhibited the complete spectrum of melanosomal profiles. Likewise the depth of tumour invasion could not be correlated with any particular melanosomal picture: i.e. deeper invading tumours did not necessarily contain more undifferentiated cells.

Series number * = pre-existing naevus	Level of invasion	Predominant cell type (in brackets are cell types which were seen occasionally)	Grade of Differentiation	MELANOSOMES							
				Spheroidal	Ellipsoidal	Normal	Abortive	Vacuolar	Granular	Lamellar	Autophagocytosis
LENTIGO MALIGNA MELANOMA											
8	2	II/III(IV)	B	+	++	++	++	-	+	-	+
9	3	IV(III)	C	+++	+	-	+	+	+	+	-
11	4	II/III	B	+	++	++	++	+	+	-	+
12	3	I/II	B	+	++	++	++	+	+	+	+
13	2	III(II)	C	+++	-	-	++	+	++	+	+
22*	2	II(III)(IV)	B	++	++	++	++	+	+	+	+
24	4	III(II)	C	+++	-	-	++	+	++	+	+
28*	5	I/II	B	+	++	++	+	+	+	-	+
29	4	III(IV)	C	++	++	+	++	+	++	-	+
30	3	II(III)(IV)	B	+	+++	++	+	-	+	+	+
34	2	I	A	+	+++	+++	+	-	-	-	+
40	4	II	B	++	++	++	++	+	+	+	+
43*	5	II(I)(III)	B	+	+++	++	++	-	+	+	+
46	3	II	B	+	+++	++	++	+	+	+	+

Table 6: Melanosomal characteristics in lentigo maligna melanoma

+++ = Nearly all ++ = Frequent + = Occasional - = Not seen

Series number * = pre-existing naevus	Level of Invasion	Predominant cell type (In brackets are cell types which were seen occasionally)	Grade of Differentiation	MELANOSOMES							Autophagocytosis
				Spheroidal	Ellipsoidal	Normal	Abortive	Vacuolar	Granular	Lamellar	
SUPERFICIAL SPREADING MELANOMA											
1	?	III	C	+++	-	-	++	++	++	++	++
5*	5	III(IV)	C	+++	-	-	+	+	++	+	+
7	3	II(III)(IV)	B	++	++	++	++	+	+	+	+
10	4	II/III	C	+++	+	+	++	++	++	+	+
14	4	III(IV)	C	+++	+	+	++	++	++	+	+
15	2	III(II)(IV)	C	+++	+	-	++	++	++	+	++
20*	2	II/III	C	+++	+	-	+++	++	++	+	++
25*	4	III(IV)	C	+++	+	+	++	+	++	++	++
27*	5	II(I)(IV)	B	++	++	++	++	+	+	-	+
33*	3	II/III	C	+++	-	-	++	++	++	+	+
35*	3	III(II)	C	+++	+	+	++	++	++	++	++
37	3	III(II)(IV)	B	+++	+	+	++	++	++	+	+
39	5	II	B	++	++	++	++	++	+	+	++
45	3	III(II)	C	+++	-	-	++	+	++	+	+

Table 7: Melanosomal characteristics in superficial spreading melanoma.

+++ = Nearly all ++ = Frequent + = Occasional - = Not seen

Series number * = Pre-existing naevus	Level of Invasion	Predominant cell type (In brackets are cell types which were seen occasionally)	Grade of Differentiation	MELANOSOMES							
				Spheroidal	Ellipsoidal	Normal	Abortive	Vacuolar	Granular	Lamellar	Autophagocytosis
NODULAR MELANOMA											
2	3	III(IV)	C	+++	-	-	+	-	++	+	+
3	5	III(II)(IV)	C	+++	-	-	++	+	++	+	+
16	4	III/IV	C	+++	+	-	+	+	++	+	++
26	4	II/III(IV)	C	++	++	+	++	+	++	+	+
38*	4	II/III	B	+++	+	+	++	+	++	++	+
41*	3	II/III	C	++	++	+	++	+	++	+	+
42	5	I/II	B	++	++	++	++	+	+	+	-
44	4	III(II)(IV)	C	+++	-	-	++	+	+	+	+
63	4	IV	C	+	+	-	+	+	+	-	-
SECONDARY MELANOMA											
31		III(II)	C	+++	+	-	++	+	++	++	+
32		III(IV)	C	+++	-	-	++	+	++	+	+
51		II(III)	B	++	++	++	++	+	++	+	+

Table 8: Melanosomal characteristics in nodular melanoma and secondary melanoma deposits.

+++ = Nearly all ++ = Frequent + = Occasional - = Not seen

Table 9 summarises the results according to histogenetic type:

Histogenetic type	Dominant cell type (Clark)	Differentiation	MELANOSOMES							Autophagocytosis
			Spheroidal	Ellipsoidal	Normal	Abortive	Vacuolar	Granular	Lamellar	
LMM	II(III)	B(C)	++	++	++	++	+	+	+	++
SSM	III(II)	C(B)	+++	+	+	++	++	++	+	++
NM	III(II)	C(B)	+++	+	+	++	+	++	+	+
Secondary Deposit	III	C	+++	+	+	++	+	++	+	+

Table 9: Summary of ultrastructural findings in 40 malignant melanosomes according to histogenetic type.

Key

LMM - Lentigo maligna melanoma

SSM - Superficial spreading melanoma

NM - Nodular melanoma

Dominant cell type - Terminology as on page 182

Differentiation - As on page 181

+++ - Nearly all

++ - Frequent

\+ - Occasional but rare

(iii) Electron microscopy dopa reaction

Ten specimens were examined:

Lentigo maligna melanoma (LMM)	4
Superficial spreading melanoma	3
Nodular melanoma	1
Local cutaneous recurrence (from LMM)	1
Secondary cutaneous deposit	1

Table 10: Electron microscopic dopa reactions carried out on malignant melanoma specimens.

Reaction product was noted in all tumours which had been incubated in dopa, but not in those incubated in buffer alone (control series).

Cellular localisation of reaction product

Although reaction product was noted in all tumours incubated in dopa it was not seen in all tumour cells. On the contrary, it was seen in the minority of tumour cells. Reaction product was never seen in melanophages even though it was often detectable in neighbouring tumour cells (Plate 87).

Subcellular localisation of reaction product

Reaction product in the tumour cells was seen in and around the Golgi apparatus in a situation identical to that noted in melanocytes irradiated with ultraviolet (see Chapter IV) i.e. in the saccules of both faces of the Golgi apparatus, in vesicles (diameter 50nm - 120nm) and endoplasmic reticulum closely related to the Golgi (GERL) and in vacuoles (diameter 100nm - 300nm) seen usually in the Golgi region (Plates 77 and 78).

In contrast to the subcellular localisation in normal melanocytes (Chapter IV) reaction product was sometimes seen lining numerous large vacuoles (diameter 400nm - 600nm) scattered throughout the cytoplasm. This occurred particularly in cells where vacuolar melanosomes were plentiful (Plates 79 and 80).

Reaction product was seldom identified in melanosomes showing any degree of structural organisation, (e.g. melanosomes with fibrillar or striated appearance, abortive and granular melanosomes) though it was often seen in and around the Golgi region of cells containing such organelles (Plates 83 and 86).

An exception to this was the identification of reaction product in patterns suggesting that it had been deposited in lamellar organelles (Plates 81 - 83).

Type IV cells (page 182 without identifiable organelles were seen to contain reaction product in the Golgi apparatus and GERL (Plate 84) and peripheral vesicles (Plate 85).

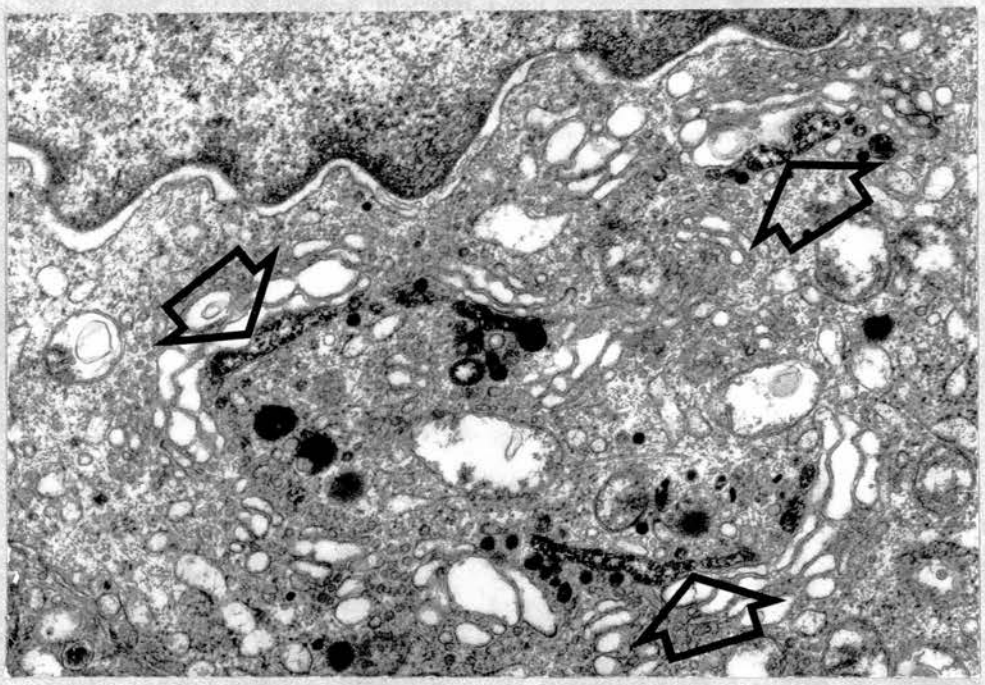


Plate 77 (X 26,000): Dopa reaction product in Golgi saccules (open arrows) and related GERL. Reaction product also seen in vesicles (diameter 200nm) near Golgi apparatus.

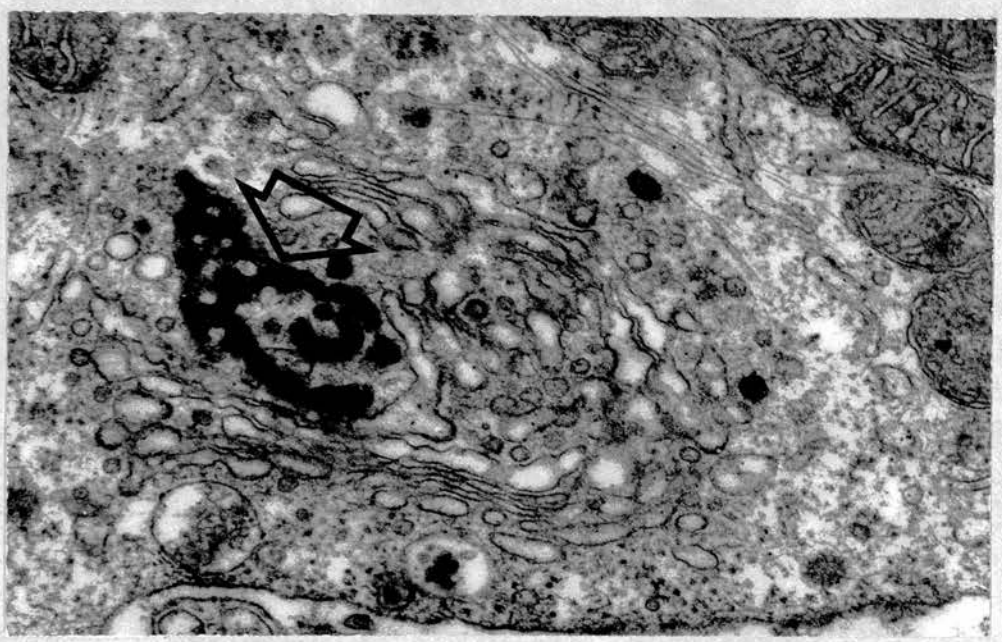


Plate 78 (X 43,000): Dopa reaction product in GERL (arrow)

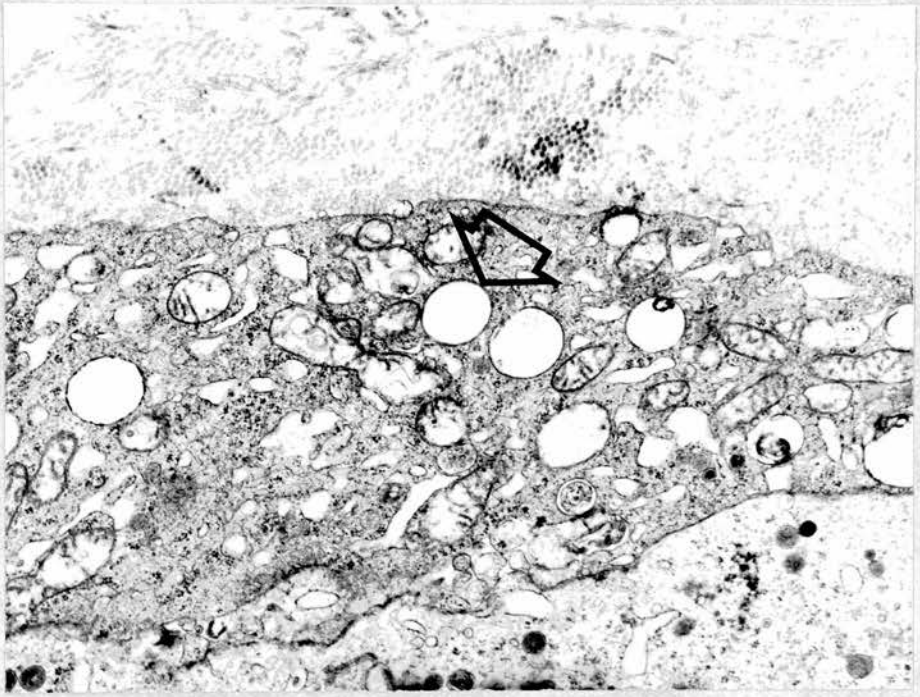


Plate 79 (X 15,000): Vacuolar melanosomes (arrow) in control. (Incubation in buffer only).

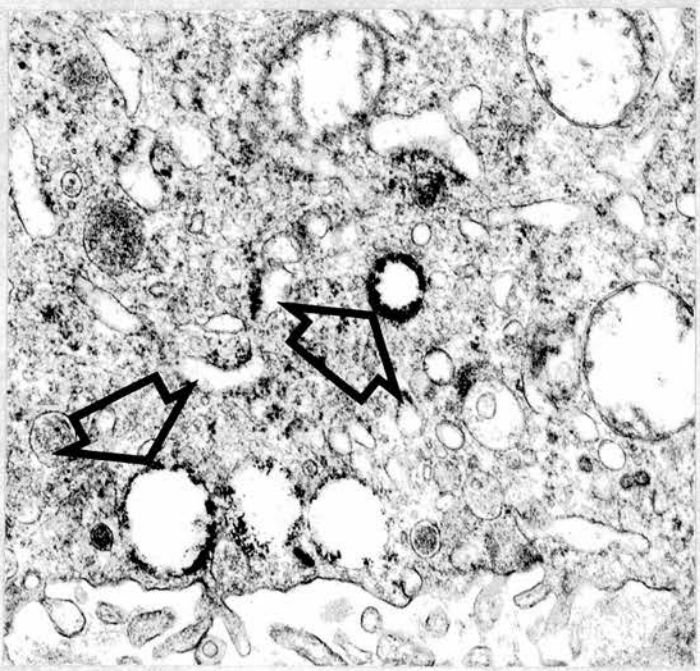


Plate 80 (X 28,000): Dopa reaction product in vacuolar melanosomes (arrow). Same tumour as above incubated in dopa.

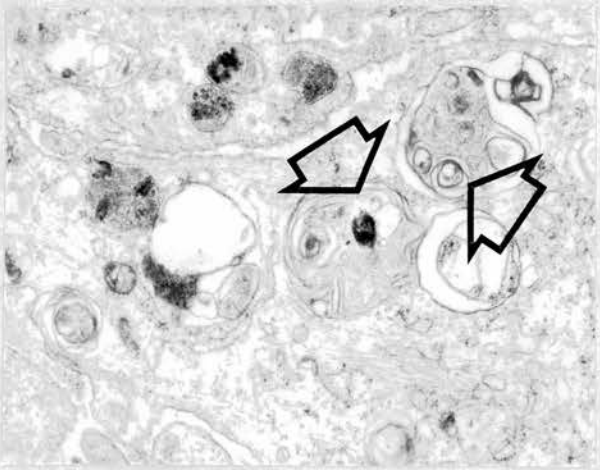


Plate 81 (X 22,000): Control reaction. Lamellar, lysosomal-like, melanosomes are arrowed. Some pigment deposition in one on left.

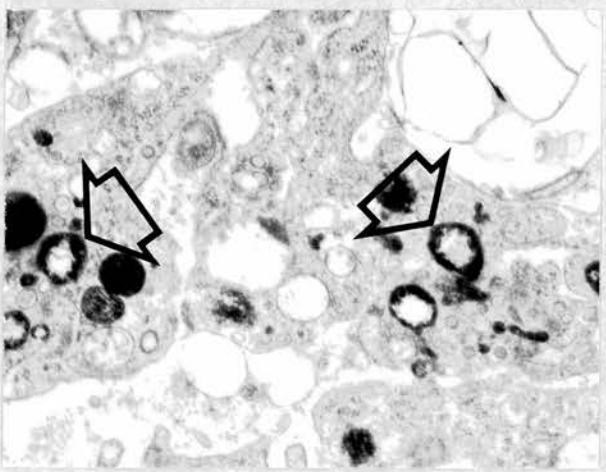


Plate 82 (X 20,000): Dopa incubation. Reaction product seen in profiles which are probably similar to melanosomes arrowed in Plate 81.

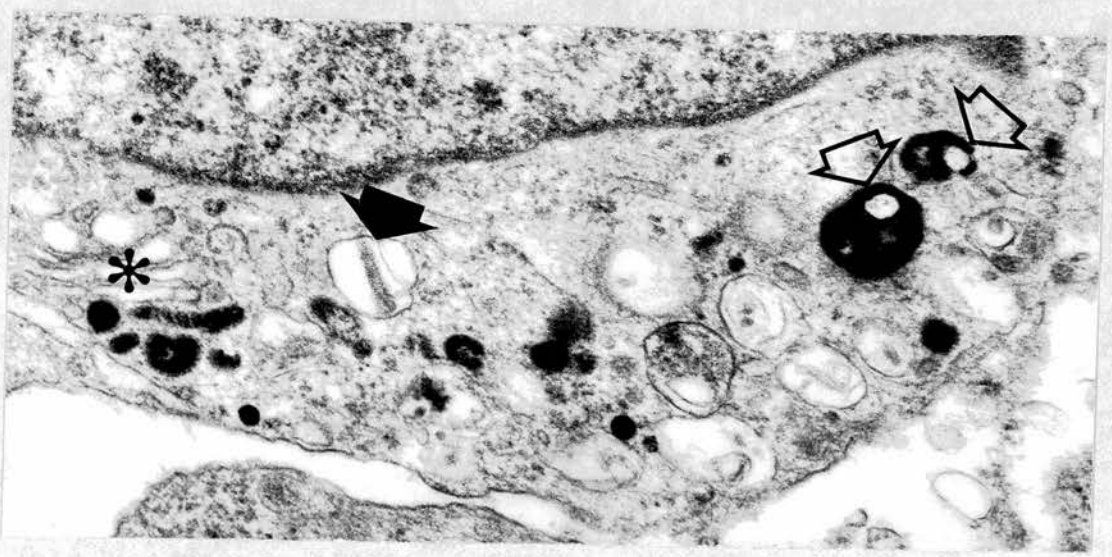


Plate 83 (X 43,000): Dopa incubation. Reaction product seen in Golgi apparatus and in vesicles nearby (*). Reaction product also in melanosomes similar to those arrowed in Plate 81 (open arrows). No reaction product in abortive melanosomes (arrow).

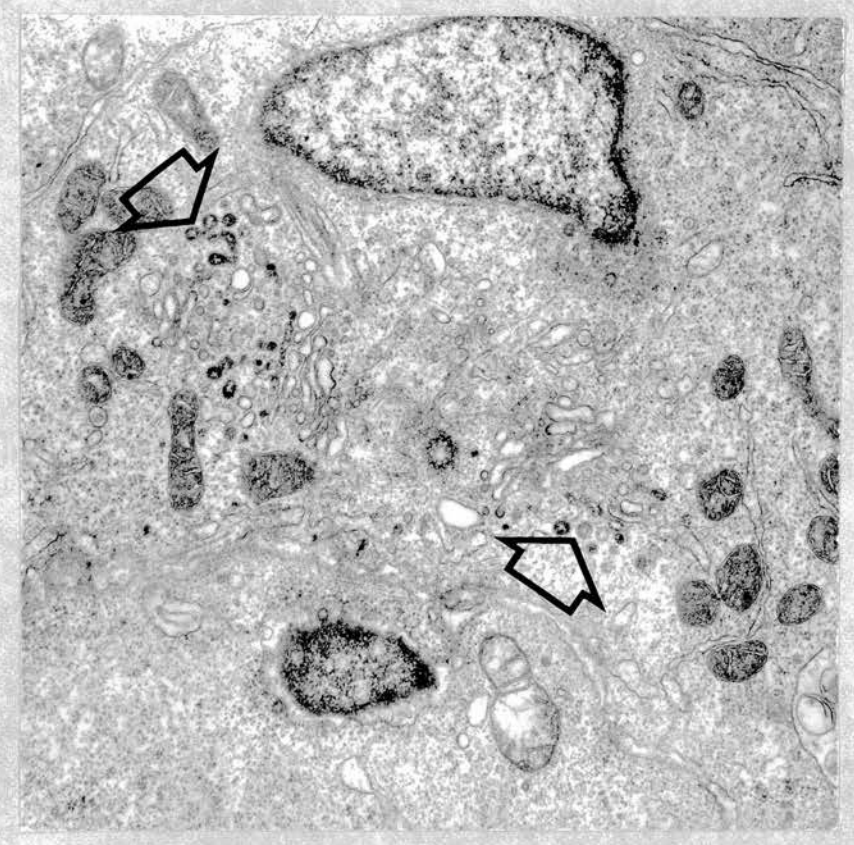


Plate 84 (X 20,000): Dopa reaction product seen in GERL of Type IV cell (open arrows). Centriole, small arrow.

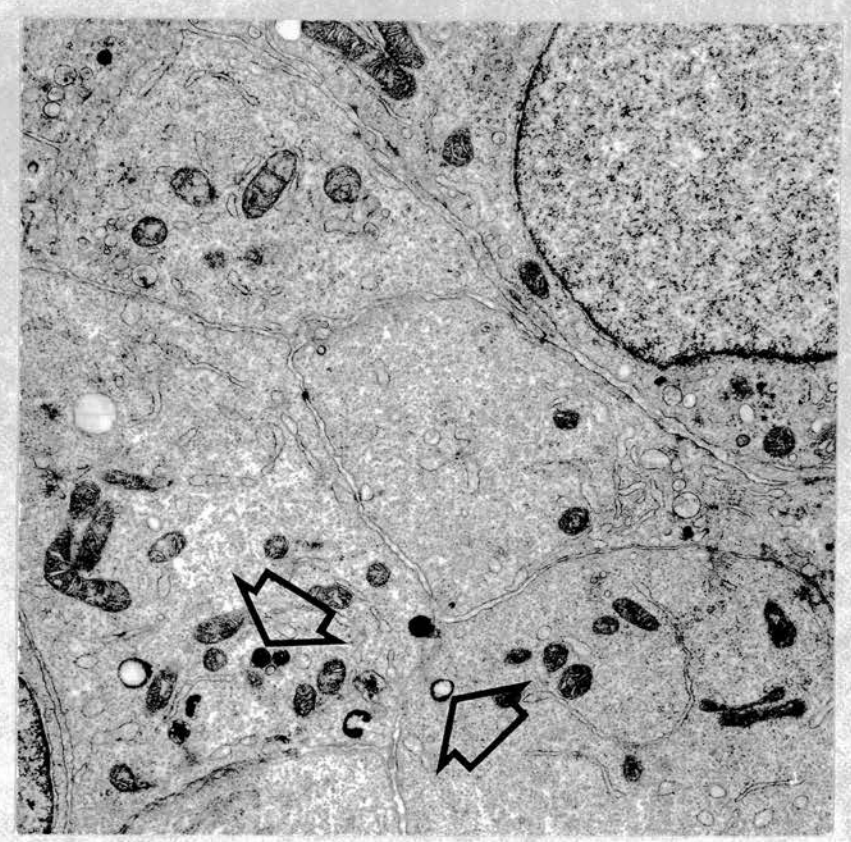


Plate 85 (X 10,000): Dopa reaction product seen in peripheral vesicles of Type IV cells (arrows).

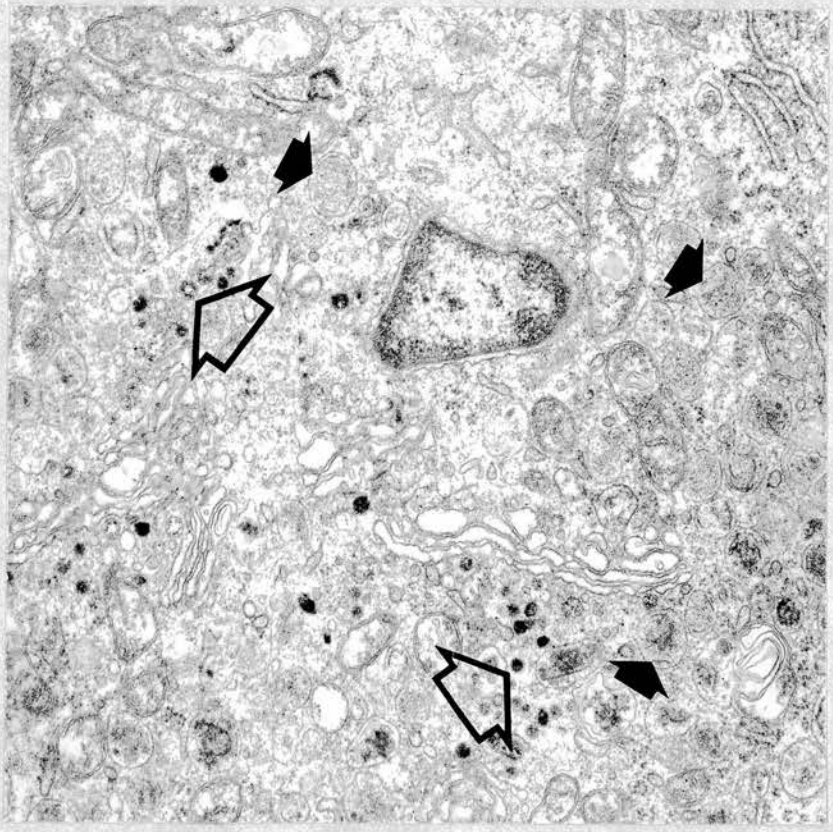


Plate 86 (X 22,000): Dopa reaction. Reaction product present in tumour cells (open arrows), but not seen in granular and abortive melanosomes (small arrows).

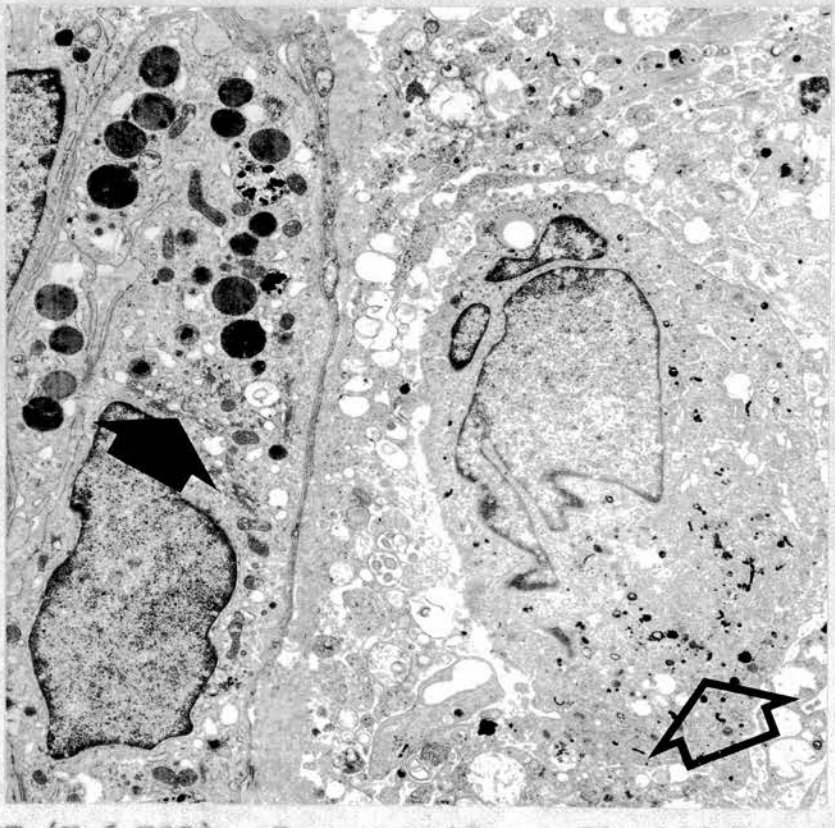


Plate 87 (X 6,500): Dopa reaction. Plentiful reaction product visible in tumour cell on right (open arrow), but none seen in melanophage on left (arrow).

The subcellular localisation of reaction product is summarised in Figure 15.

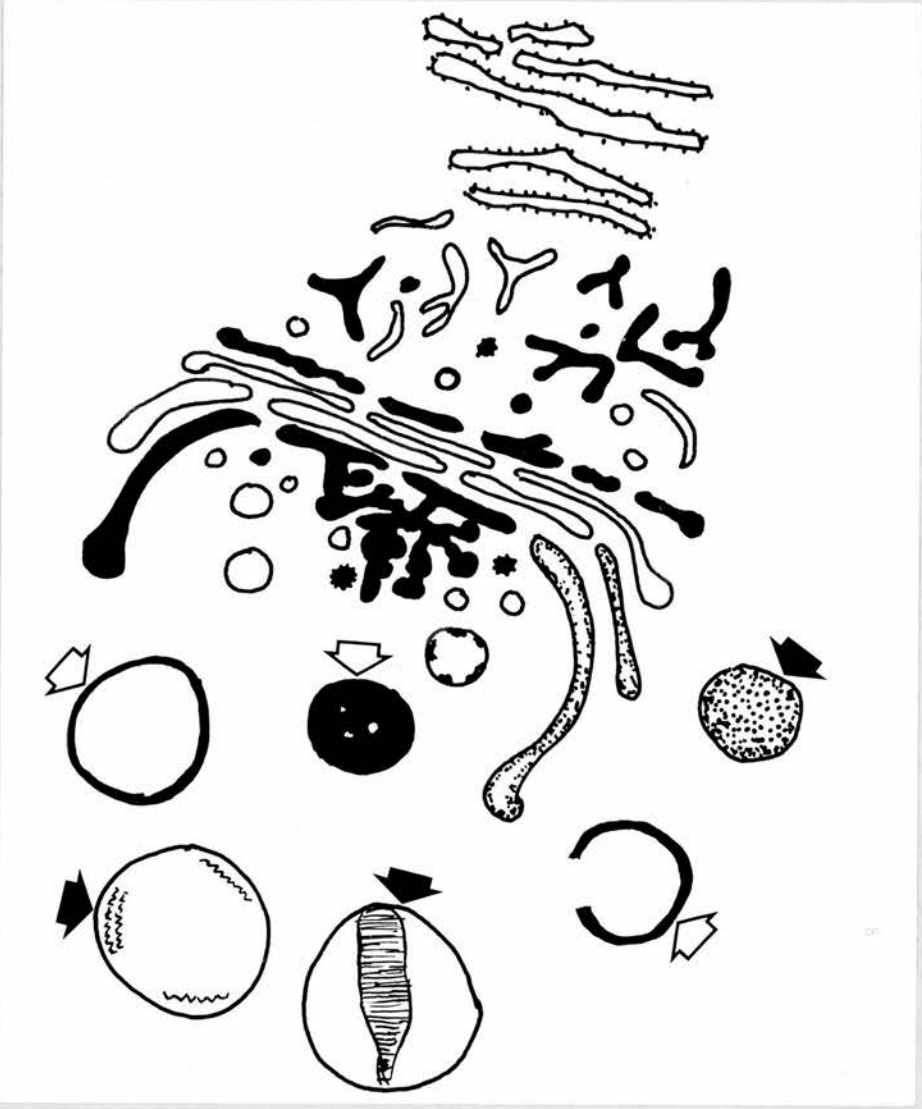


Figure 15: Subcellular localisation of dopa reaction product in malignant melanoma.
Reaction product is seen in the Golgi saccules and GERL. It is also noted lining some vacuolar profiles and is present in occasional lamellar melanosomes (open arrows). It is rarely seen in granular and abortive melanosomes (arrows). See Figure 11 for comparison.

4. DISCUSSION

a) Findings in this study

The above results indicate that the ultrastructure of lentigo maligna melanoma is fairly characteristic. The evidence suggests that this is a well differentiated tumour composed of cells showing malignant features (in particular large convoluted nuclei and abnormal nucleoli) but involved in relatively normal melanogenesis.

In most cases this picture is in direct contrast to that seen in superficial spreading, nodular and secondary melanomas. Here anarchy displaces order (Cesarini, 1971) and melanogenesis not only occurs in abnormal spheroidal organelles, but becomes totally haphazard and disorganized. There are only minimal differences in the ultrastructure of superficial spreading, nodular and secondary melanomas. The incidence of abortive, granular and lamellar organelles seems similar in each though, in this series, vacuolar melanosomes appear to be more common in superficial spreading melanoma. It will be interesting to see whether this finding can be confirmed by others.

The fine structural appearances can therefore be classified into two broad categories. In the first the tumour cells still resemble normal melanocytes (differentiated group), whilst in the second all such resemblance is lost, and subcellular dedifferentiation is obvious (dedifferentiated group).

The existence of a preceding naevus and the depth

of invasion of the tumour cannot be related specifically to either picture. Melanomas which develop from naevi can exhibit a fine structure characteristic of either group, and the more deeply invading tumours do not appear to contain only dedifferentiated cells.

The dopa reaction provided some interesting information. The presence of reaction product in vacuolar profiles (diameter 200 to 600nm) confirmed the impression that such organelles were indeed melanosomes without structural contents. It also confirmed that this type of dopa reaction will not detect tyrosinase once it is combined with the organised structural matrix of the melanosome. Tyrosinase in this situation is apparently inactivated by the glutaraldehyde used in the prefixation (see page 104).

Interestingly, tyrosinase still seemed detectable when it was associated with the membranes of some lamellar melanosomes; it may be that the enzyme is fixed to these membranes in a way similar to its attachment to vesicular and vacuolar membranes, where it is readily identifiable by the dopa reaction.

The dopa reactivity of the Golgi and GER regions in Type IV cells should spell caution in interpreting all these cells as undifferentiated (Clark et al., 1972). The dopa reaction demonstrates that some are indeed synthesizing tyrosinase. Some may therefore be young tumour cells which have not yet formed melanosomes. This could explain their occasional presence in even the most differentiated of tumours (e.g. patient numbers 8, 22 and 30. Table 6).

The prognosis in patients with malignant melanoma depends on many variables, but can be conveniently related to three important features, stage (spread) of the disease (Luce et al., 1973), depth of invasion of primary tumour (Clark et al., 1969) and histogenetic pattern (Clark et al., 1969). Only the last two will be discussed here as only they are relevant in this study.

The level of invasion of the tumour nodule is important because of its direct correlation with prognosis (Clark et al., 1969; McGovern, 1970 and Little, 1972). The figures quoted by Clark et al. (1969) are summarised in Table 11.

Depth of invasion of primary tumour	% Survivors (5-7 yr follow up)
Level II	72
Level III	47
Level IV	32
Level V	12*

* Only 3 year follow up

Table 11: % survival in 208 patients with malignant melanomas related to depth of invasion of primary tumour. (Information from Clark et al., 1969)

The series of Clark et al. (1969) also highlighted the relationship between histogenetic type and prognosis: Table 12.

Histogenetic type	% survivors (208 patients - followed up 5-7 years)
Lentigo maligna melanoma	55
Superficial spreading melanoma	46
Nodular melanoma	27

Table 12

(Information from Clark et al., 1969)

The figures reported by McGovern (1970), Huves et al. (1973) and Jourdain (1974) show similar trends.

It is, of course, too early to relate the ultrastructural appearances noted in this study with prognosis. There is no clear cut relationship between fine structure and depth of invasion of the primary tumour (see above). Nevertheless it is possible that those patients with tumours extending to a depth of Level V may have a better prognosis if the ultrastructural appearance is that of the differentiated group (e.g. patients numbers 27, 28 and 43, Tables 6 and 7, and 42 Table 8).

Put another way, the 12% of patients surviving with primary tumours at Level V (Table 11) could be those with tumours having a differentiated ultrastructural appearance.

The same sort of reasoning can be applied to survival according to histogenetic patterns. Do those patients who survive with nodular melanoma (27% in Table 12) have ultrastructurally differentiated tumours? Conversely, are most of the deaths associated with the dedifferentiated pattern? Time is needed before these important questions can be answered. The series should be analysed again in five years, and these points investigated. If correlations are found, then there could be obvious therapeutic implications, as more aggressive treatment is indicated in patients with a poor prognosis.

b) Comparison of results in this study with those of others

It is now worthwhile comparing the results in this series with those in others. As mentioned in the introduction, the number of patients studied by some workers was exceedingly small. The results of this study show that, with such small numbers, the exception rather than the rule could have been reported, so caution is indicated when drawing conclusions.

The number of patients studied in published work is recorded below.

Anton Lamprecht et al., (1971)	- 1 (LMM)
Arao et al., (1971)	- 2 (LMM)
Cesarini, (1971)	- 100 (no details of histogenetic type)
Clark et al., (1972)	- 7 (LMM-3, SSM-4)
Clark and Bretton, (1972)	- No numbers stated
Jakubowicz et al., (1970)	- 2 (no details of histogenetic type)
Hirone et al., (1971)	- 6 (LMM-2, NM-2, Preceding naevus-2)
Klingmuller et al., (1970)	- No numbers stated
Klug and Günther, (1972)	- 32 (no details of histogenetic type)
McGovern and Lane Brown, (1969)	- No numbers stated
Mishima, (1967)	- 13 (Malignant naevocytoma-11, malignant melanocytoma-2)
Mishima et al., (1975)	- 23 (SSM-23)
Lupulesco et al., (1973)	- 2 (LMM-2)

The two largest series (Cesarini, 1971 and Klug and Günther, 1972) are unhelpful. Klug and Günther (1972) distinguish two main types of cell in malignant melanoma which they term "Type A" and "Type B". Type A cells contain cigar shaped melanosomes, and Type B cells spheroidal ones. Unfortunately they do not correlate the occurrence of these types of cells with different histogenetic patterns, but there is a suggestion that

their Type B cells correspond with the cells noted in lentigo maligna melanoma by Mishima (1967). This is a pity as, until then, their Type A cell appeared to correspond with the differentiated type of cell described in this Chapter, and their Type B cell with the dedifferentiated type.

The descriptions in Cesarini's (1971) study of 100 cases of malignant melanoma are too vague, and there was no attempt to correlate melanosomal profiles with histogenetic types. However Fig. 4c does show some typical vacuolar melanosomes.

The findings in this series are in general agreement with those of Clark et al. (1972). This is particularly encouraging as the terminology used here was based on that suggested by this group of workers. They also emphasized the overlap between the fine structural appearances seen in the different histogenetic types. This has been verified, but the main conclusion that lentigo maligna melanoma usually has a distinctive ultrastructure still seems valid. In addition to the findings of Clark et al., the vacuolar type of melanosome was also described and emphasized in this study, especially as it appeared to be more common in superficial spreading melanoma.

Similar findings in lentigo maligna melanoma have been reported by Hirone et al. (1971) and Lupulesco et al. (1973), and these workers also comment on the

relatively normal appearance of the melanosomes in this histogenetic type. In contrast Mishima (1967) and McGovern and Lane Brown (1969) referred to the pleomorphic appearance of the melanosomes in lentigo maligna melanoma. However the observations of Mishima could have been due to the fact that they were made at a time when superficial spreading melanoma had not been distinguished from lentigo maligna melanoma, and were therefore based on the study of two histogenetic types.

McGovern and Lane Brown (1969) and Anton Lamprecht et al. (1972) have drawn attention to large cytoplasmic vacuoles (diameter 1 - 1.4μm) in the neoplastic cells of lentigo maligna melanoma. Similar vacuoles have been noted in the series described in this Chapter, though they have been seen as often in superficial spreading and nodular melanomas. The view that they are due to lipid degeneration (Anton Lamprecht et al., 1972) is supported. Their appearance is quite different from that of vacuolar melanosomes (Page 186).

Mishima and Matsunaka (1975) have recently described their findings in superficial spreading melanoma. They considered that the neoplastic cells in this type of melanoma synthesized "rather uniquely spheroid melanosomes containing finely granular, densely distributed melanin particles and occasionally whirlpool-like internal structure", and they record the diameter of these

melanosomes as 500 - 700nm. The findings reported here are different. The granular and lamellar melanosomes are found frequently in melanomas other than superficial spreading type, and numerous measurements indicated that their diameter was smaller, i.e. 150 - 400nm. Studies on more specimens of this histogenetic type are necessary before any firm conclusions can be drawn.

Klingmuller et al. (1970) were unable to relate the fine structural appearance of melanosomes with any particular histogenetic type, and Jakubowicz et al. (1970) felt that the study of melanosomal morphology in malignant melanoma was of limited value, and that the differences in melanosomal structure were not great enough to afford diagnostic or differential diagnostic criteria.

c) <u>The significance of fine structure in relationship to the pathogenesis of malignant melanoma</u>

Studies on the fine structure of melanosomes in malignant melanoma stem from the original work of Mishima (1960 and 1967). On the basis of clinical and pathological differences he suggested that melanomas arise either from malignant transformation of normal melanocytes (melanocytic malignant melanoma) or from transformation of junctional naevus cells (naevocytic malignant melanoma). He supported this hypothesis

with evidence (which now appears to have been inaccurate) suggesting that melanocytic and naevocytic malignant melanomas could be distinguished by the distinctive fine structure of their melanosomes. Although Mishima's original work may be open to question, there can be no doubt that his ideas led to much further study of melanosome polymorphism as a tool in the differential diagnosis of histogenetic types of malignant melanoma.

Recent investigations into the pathogenesis of malignant melanoma have provided little support for Mishima's views. Lever (1975) has pointed out that his hypothesis implies that all malignant melanomas other than malignant melanocytomas (in which group Mishima places lentigo maligna melanoma) develop from naevi. Since lentigo maligna melanomas comprise little more than 10% of all malignant melanomas, this would mean that 90% of all melanomas develop from pre-existing naevi. This is quite contrary to the present belief that only 20% of malignant melanomas arise from pre-existing naevi (Clark et al., 1969). Mishima's hypothesis is also based on the assumption that the melanocyte and junctional naevus cell are two different types of cell, a view which is not supported by the ultrastructural studies of Gottlieb et al. (1965) and Hirone et al. (1971). McGovern and Lane Brown (1969) also consider that "junctional naevus cells are proliferated normal melanocytes".

The histogenetic typing currently favoured by most workers (and used in this Chapter) stems from the studies of Clark (1967) and McGovern (1970). Clark et al. (1972) have found naevi associated with all three histogenetic types: lentigo maligna melanoma, superficial spreading melanoma and the nodular type. They consider that the melanocytes, whether dispersed in a pre-existing naevus or whether dispersed as part of the normal intra-epidermal melanocytic population, may be effected by the agent responsible for malignant change.

It is always difficult to be sure of the nature of any pre-existing pigmented lesion, and the criteria used in the study reported in this Chapter were probably over-strict. Nevertheless the finding that pre-existing naevi occurred with similar frequency in all histogenetic types supports the view of Clark et al. (1972). It was also evident that there was no distinctive melanosomal pattern in melanomas developing from pre-existing naevi.

Mishima's view, that melanomas developing from naevi contain characteristic melanosomes, has received support from only one group of workers recently. Hirone et al. (1971) felt that granular melanosomes were seen only in naevocytic melanomas, but only two such specimens were studied. However, in this study, it was noted that granular melanosomes can be seen in all histogenetic types of malignant melanoma and cannot be related to the presence of pre-existing naevi.

It is now quite evident (see introduction in this Chapter) that there are readily identifiable clinical and histological differences which distinguish the histogenetic types of malignant melanoma. They are, indeed, the raison d'être of the classification. For instance lentigo maligna melanoma has been shown to be slowly growing and relatively benign; whereas the other types of melanoma are much more agressive (Clark, 1967). Paralleling the clinical differences, the slower growing lentigo maligna melanoma retains the capacity for relatively normal melanogenesis whereas the more agressive tumours show little tendency to form normal melanosomes.

Moyer (1963) has shown that melanosomal fine structure is under genetic control. I_n mice, mutant alleles have been shown to cause disorientation of melanosomal fibres associated with reduction of fibre cross-linking. Although the carcinogenic stimulus in malignant melanoma remains unknown, it seems quite possible that the similar type of disorganization of melanosomal structure, noted here in superficial spreading and nodular melanomas, could be due to a somatic mutation or viral incorporation into the genome of the cell.

The contrasting biological behaviour, histology and fine structure of lentigo maligna melanoma, when compared with other types of melanoma, suggest a different mode of pathogenesis in lentigo maligna melanoma. However it remains a matter of conjecture whether this is due to a different carcinogenic agent or to a more orderly and conditioned host response to the same agent.

Chapter IX

PSEUDO-MELANOMA

Including

1. INTRODUCTION

2. MATERIAL AND METHODS

3. CASE REPORT
   a) History and examination
   b) Macroscopic picture
   c) Light microscopy
   d) Electron microscopy

4. DISCUSSION

1. INTRODUCTION

In this chapter an extreme rarity will be considered. During the search for surgically excised specimens of malignant melanoma, tissue from a pigmented breast lesion, thought on clinical grounds to be a malignant melanoma, was acquired. At operation, and on frozen section examination, it became obvious that the lesion was due to a carcinoma of the breast invading the overlying epidermis, and causing a remarkable pigmentary reaction. The opportunity of studying the fine structure of this lesion was too good to miss, and the curious picture is described below.

2. MATERIAL AND METHODS

As outlined in Chapter II.

3. CASE REPORT

a) History and examination

Mrs. A.W., aged 76 years, had noticed a pigmented lump under her right breast. It had been present for about six months and had neither bled nor changed colour. It was enlarging slowly.

Examination revealed a black pigmented nodule measuring $0.8cm^2$, situated in the submammary skin crease of the right breast fold. The nodule was surrounded by

an area of induration measuring 2.5 X 1.2cm and, deep to it, a firm irregular mass of about 3cm^2 could be felt. 0.5cm medial to the lesion was a violaceous to pink dome shaped papule measuring 0.5 X 0.3cm (Plate 88). There were two enlarged glands in the right axilla.

The lesion was removed locally under general anaesthetic (Plate 89) and, after the diagnosis of an anaplastic carcinoma of the breast had been made on frozen section, simple mastectomy was performed.

The patient was treated with post operative radiotherapy, and was alive and well in March 1976, with no evidence of tumour recurrence.

b) Macroscopic picture of excised lesion (Plate 89)

Even without histological confirmation it was obvious that the main lesion was due to a carcinoma of the breast which had invaded the overlying epidermis.

The main tumour mass was about 3cm deep to the skin surface and measured 1.5cm^2. It was invading the surrounding fat, and there was a moderate fibrous reaction.

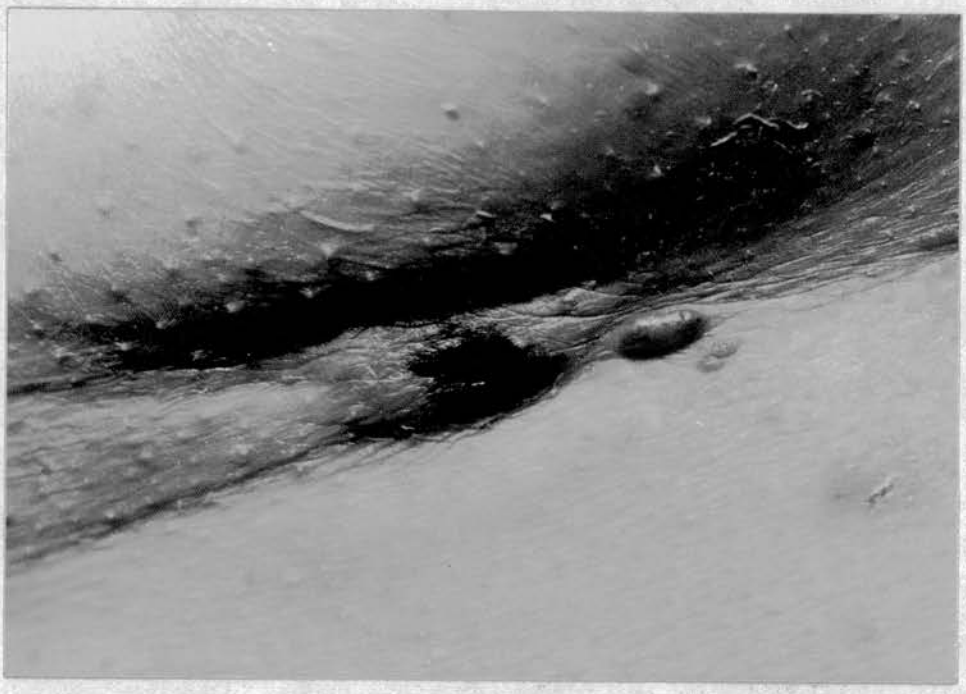


Plate 88: "Pseudo-melanoma" Right inframammary breast fold.

Plate 89: "Pseudo-melanoma" Sectioning of excised specimen reveals underlying breast carcinoma (large arrow). Small arrow indicated pigmented lesion on surface.

c) Light microscopy

Dr. A. Shivas (Department of Pathology, University of Edinburgh) reported the histological sections:

"The tumour nodule has the structure of an anaplastic carcinoma of the breast showing a prominent scirrhous reaction with marked elastosis. The overlying epidermis is infiltrated by tumour cells and considerable quantities of melanin are present in the superficial dermis. The appearances are not those of melanoma."

Several lymph nodes of the axillary tail were also replaced by cords of poorly differentiated carcinoma.

Plates 90 and 91 are photographs of haematoxylin and eosin sections of the superficial part of the lesion. In Plate 90 it can be seen that the tumour cells are dispersed in a mainly horizontal fashion in the middle and deep dermis, whereas in the superficial dermis they form cords running perpendicular to the epidermis. A picture, resembling "junctional activity" in naevi, is produced where the tumour cells reach the epidermis (Plate 91). Even in the haematoxylin and eosin sections considerable pigmentation in both the base of epidermis and superficial dermis is evident (Plate 91). In the papillary dermis occasional pigmented cellular processes can be seen surrounding tumour cells (Plate 91) though this is much more obvious in the section, depicted in Plate 92, stained for melanin (Masson Fontana).

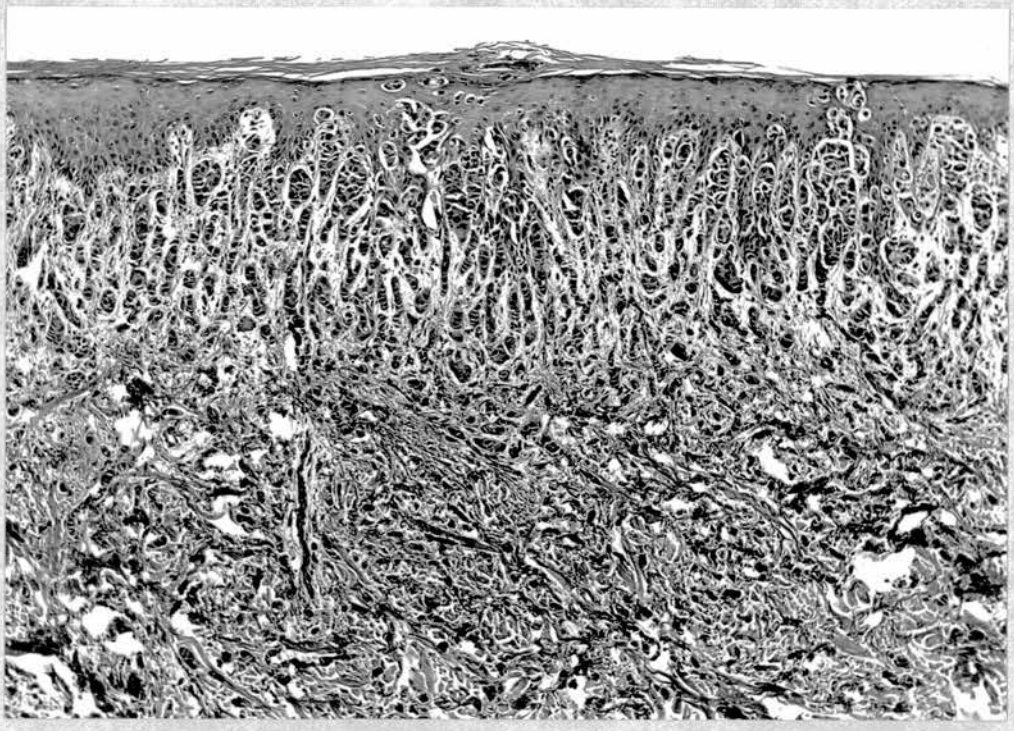


Plate 90 (X 70): "Pseudo-melanoma" The tumour cells are scattered in a random manner in the mid dermis, but in the superficial dermis they group in columns perpendicular to the skin surface. There is some epidermal invasion by tumour cells. (H and E)

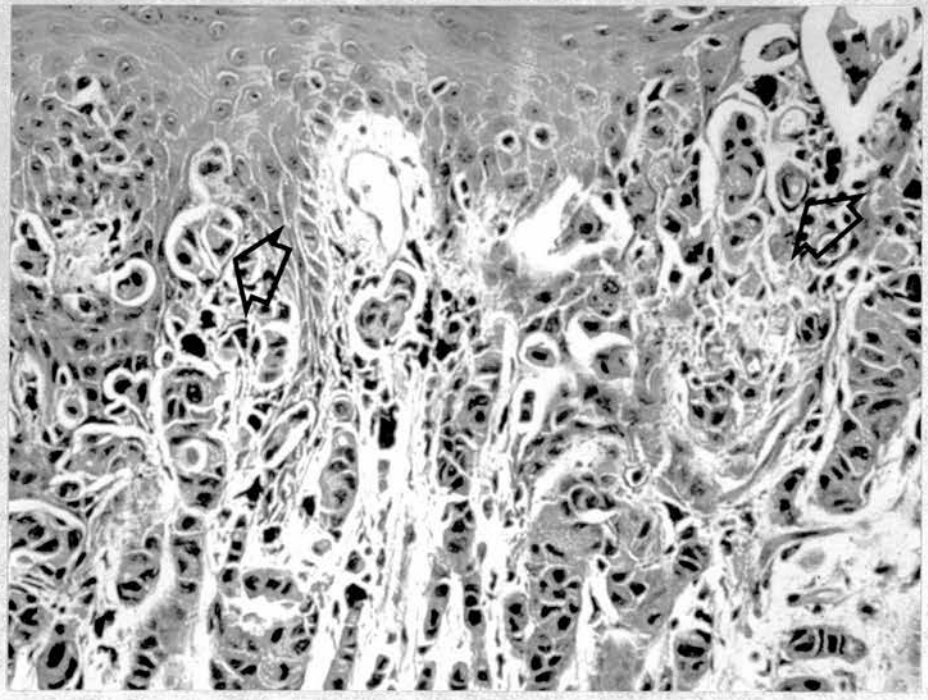


Plate 91 (X 400): "Pseudo-melanoma" Papillary dermis showing picture simulating junctional activity. On careful inspection pigmented dendrites can be seen surrounding some tumour cells (arrows). (H and E)

d) Electron microscopy

There was considerable hyperpigmentation in keratinocytes of the basal and suprabasal cell layers. The epidermal melanocytes had numerous dendritic processes and were packed with melanosomes at all stages of development. The structure of the melanosomes appeared normal; so the melanocytes could be called Type I cells using the terminology outlined on page 182 (Plate 93).

Cells of the breast carcinoma (Plate 98) looked undifferentiated. They had convoluted nuclei and often prominent nucleoli. Free ribosomes and mitochondria were abundant in their cytoplasm and occasionally vacuoles, due to presumed lipid degeneration, were noted (Plates 94 and 95). Many cells contained bundles of fine fibres (Plates 95 and 96).

The most interesting part of the lesion was that in the papillary dermis (i.e. that area shown in Plates 91 and 92). Here melanocytes became intimately associated with tumour cells, and their dendritic processes, packed with melanosomes, surrounded them (Plates 94, 95 and 96). Melanocytes were even seen surrounding tumour cells in the mid dermis. Although minor phagocytosis of melanosomes by tumour cells was noted occasionally (Plates 95 and 96), no signs of phagocytosis of tumour cells by melanocytes were evident. There were, however, numerous pigment laden melanophages scattered throughout the dermis (Plate 97).

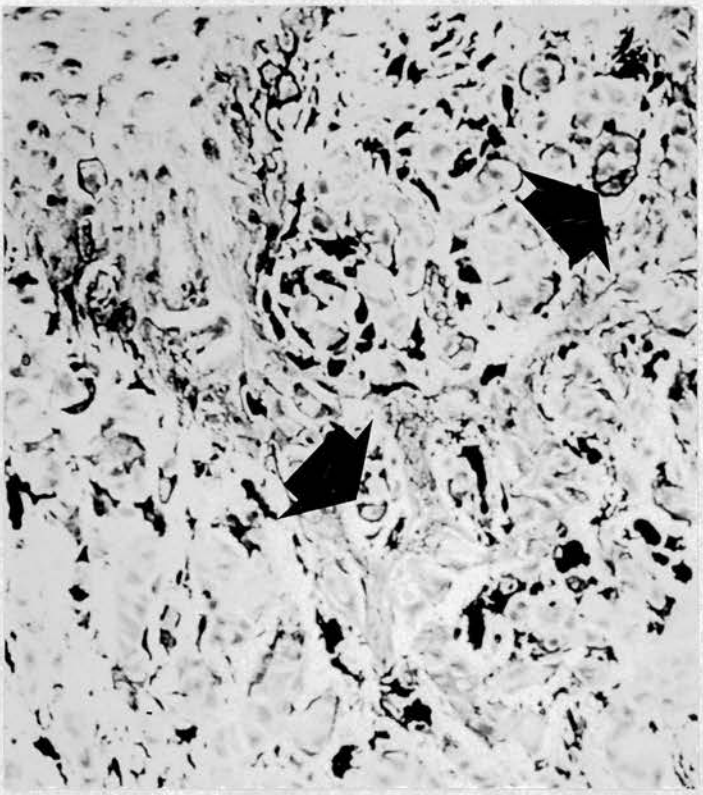


Plate 92 (X 400): "Pseudo-melanoma" Papillary dermis. Melanin stain reveals numerous pigmented dendritic cells amongst tumour cells. Arrows indicate processes surrounding tumour cells (Masson-Fontana)

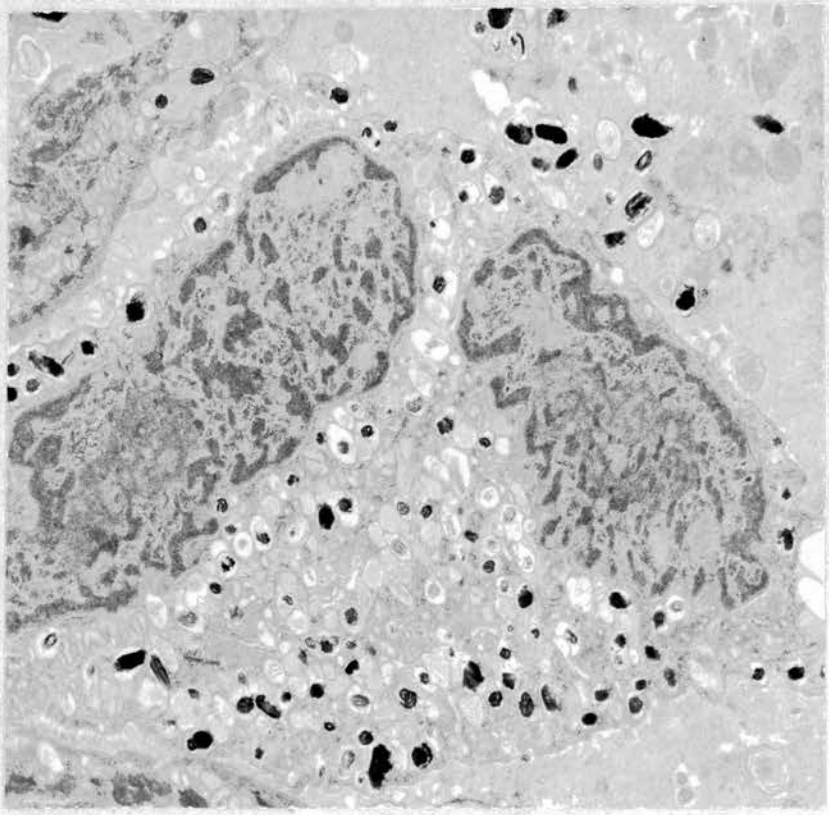


Plate 93 (X 13,500): "Pseudo-melanoma" Cell body of melanocyte in epidermis. Note that it contains numerous melanosomes in all stages of relatively normal development.

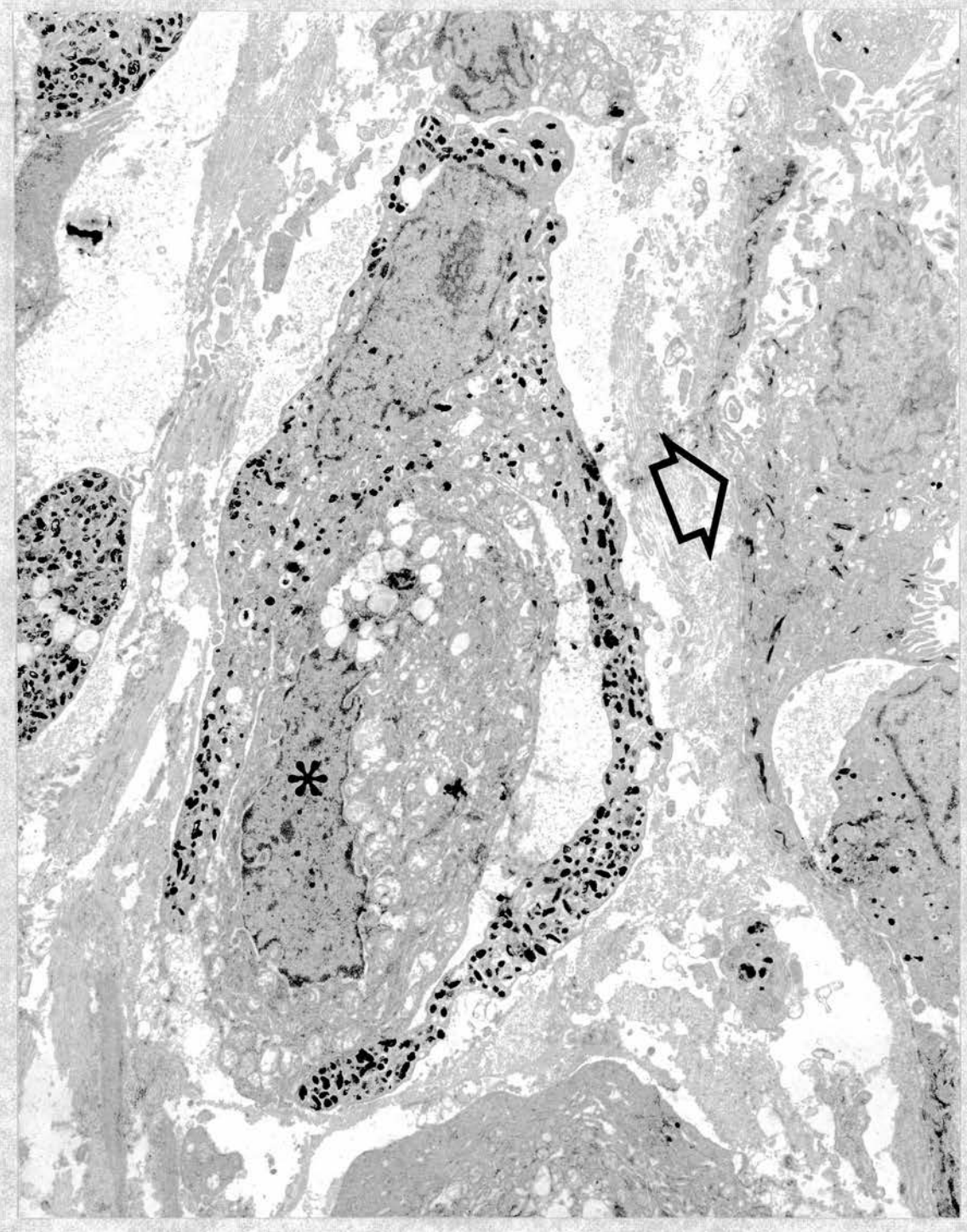


Plate 94 (X 6,500): "Pseudo-melanoma" Just below the epidermal basal lamina (arrow) a melanocyte, packed with melanosomes, surrounds a tumour cell (*).

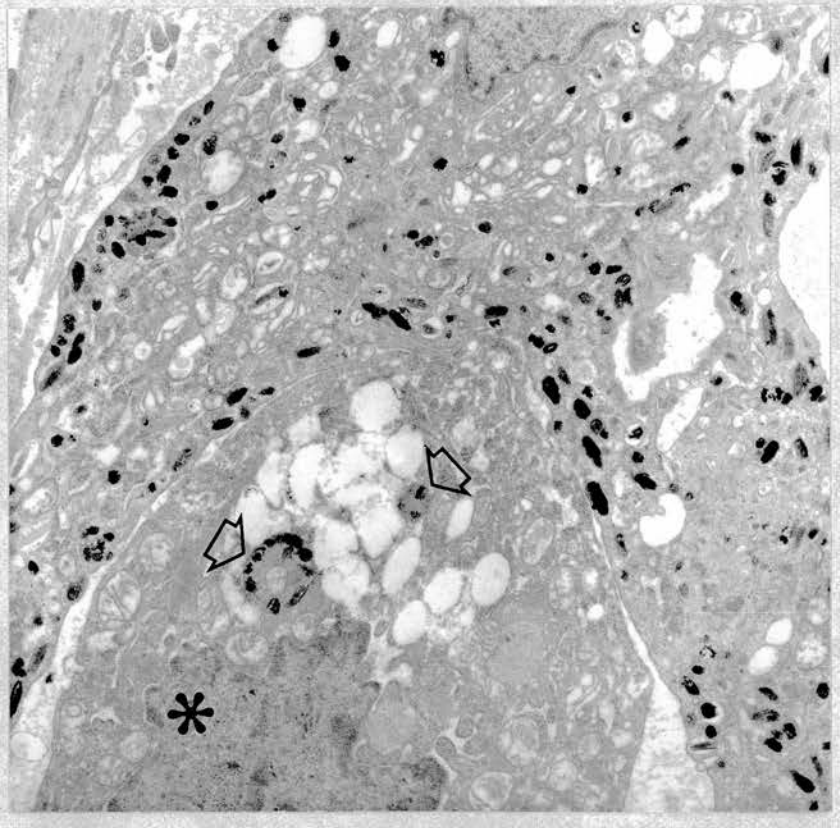


Plate 95 (X 9,500): "Pseudo-melanoma" Tumour cell (*) with vacuoles (lipid extraction) and melanocyte in apposition. Note two phagosomes containing melanosomes in tumour cell (arrows).

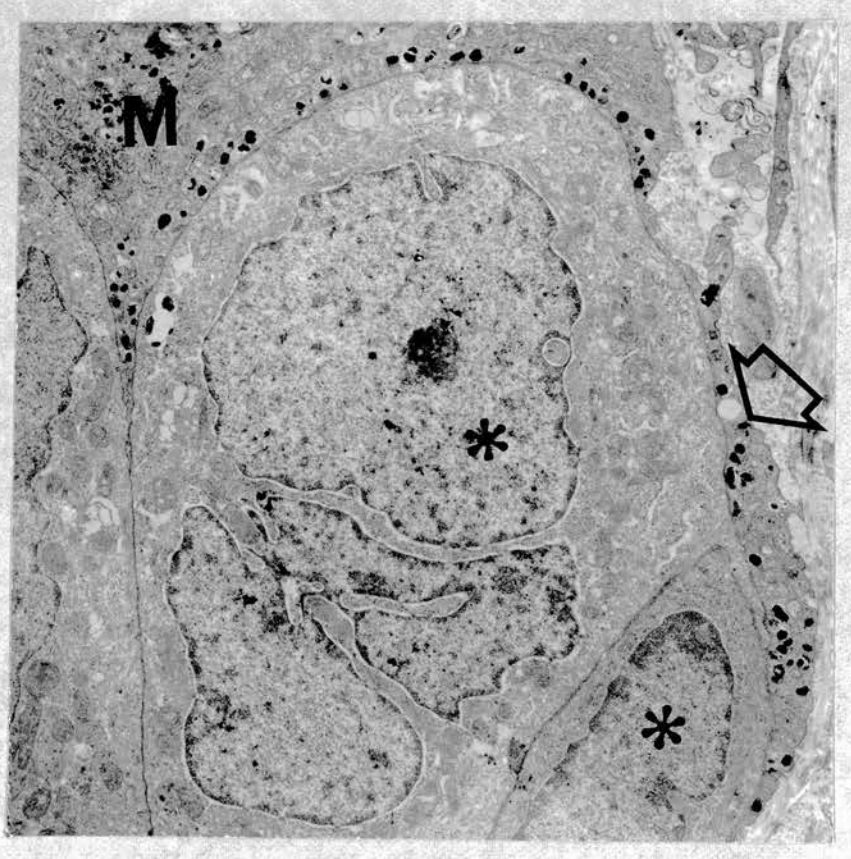


Plate 96 (X 6,000): "Pseudo-melanoma" Dendritic process (arrow) of melanocyte (M) hugging tumour cells (*)

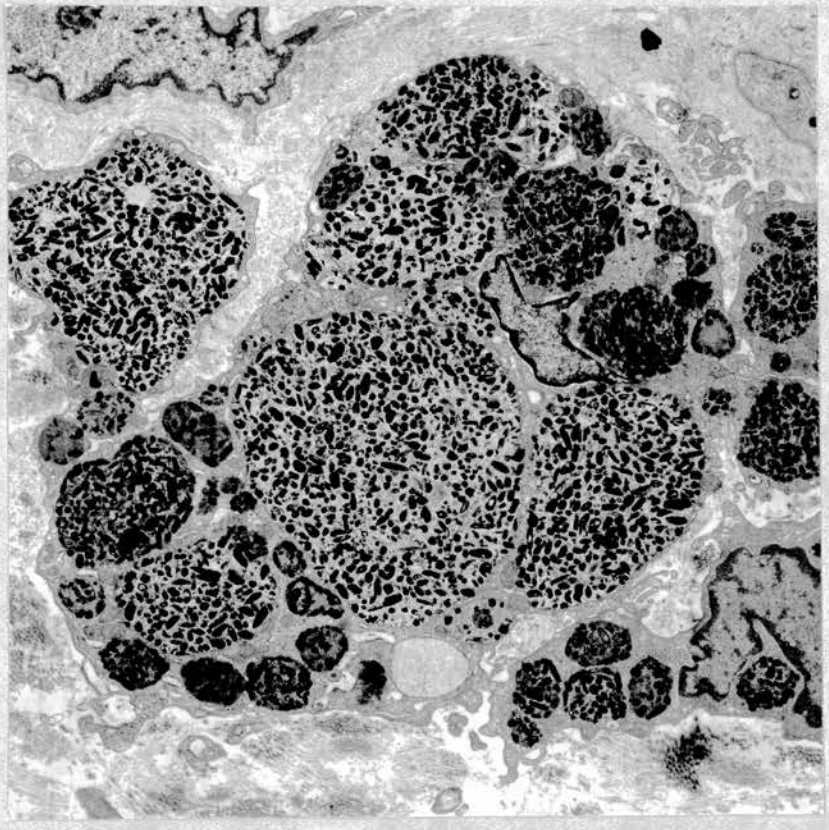


Plate 97 (X 5,500): "Pseudo-melanoma" Melanophage in papillary dermis packed with phagocytosed melanin. Tumour cells in same region seldom contained melanin.

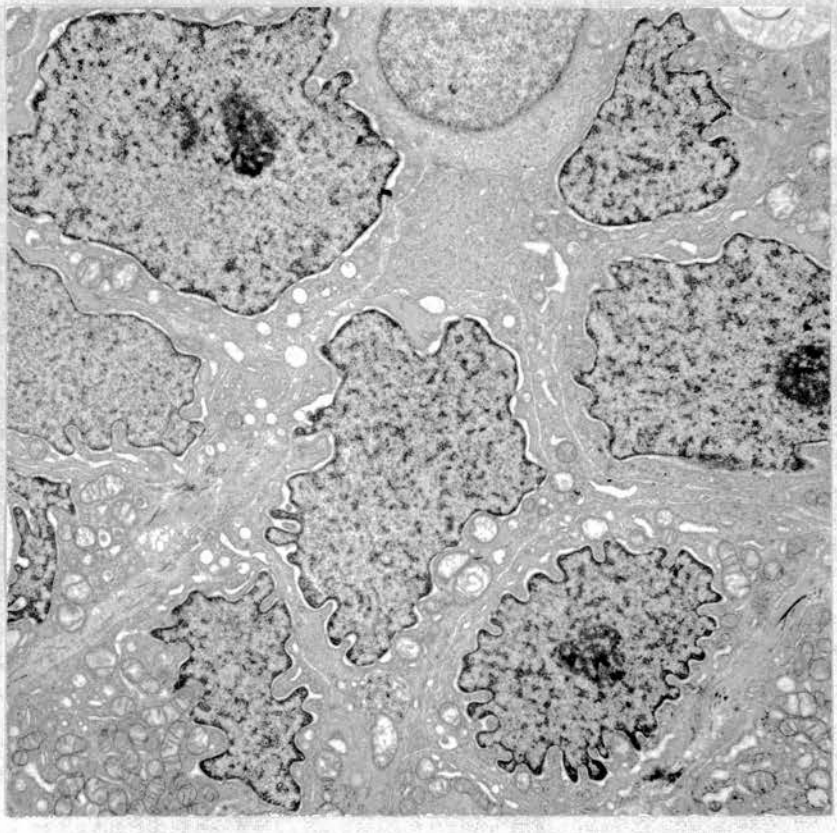


Plate 98 (X 6,000): "Pseudo-melanoma" Tumour cells in main tumour. (specimens taken from region indicated by arrow in Plate 89). Note that melanocytes are not seen.

4. DISCUSSION

In 1926 both Masson and Caudiere referred to single cases of carcinoma of the breast invading the overlying skin and producing a bizarre response in the melanocytes of the epidermis. Lewis has seen the phenomenon more frequently in Africans, and a light microscopic illustration of it appeared in a recent publication (Copeman et al., 1973) with a comment that the "very striking appearance is likely to cause confusion with malignant melanoma." This seems to be the first ultrastructural study of the response.

Little is known about the mechanisms which influence the normal melanocyte to remain in its intraepidermal location. However it does seem likely that keratinocytes are involved in some way and Prunieras (1969) has suggested that keratinocytes have to be present if melanocytes are to multiply *in vitro*. Whatever the mechanism, it seems an inescapable fact that there is a close symbiotic relationship between melanocytes and surrounding keratinocytes in both normal and abnormal conditions (Pinkus et al., 1959).

Some epidermal tumours, for example basal cell carcinoma and basal cell papilloma, (more often known to dermatologists as "seborrhoeic wart") are frequently pigmented (Zelickson, 1967 and Lever, 1975). In addition to the proliferation of keratinocytes these tumours contain many functional melanocytes (Bleehen, 1975). In basal cell papillomas the melanin pigment

is found predominantly in the tumour cells (Lever, 1975), while it is seen in both melanocytes (Zelickson, 1967 and Bleehen, 1975) and tumour cells (Ishibashi et al., 1971) in basal cell carcinomas. Mishima and Pinkus (1960) drew attention to the increased number of highly dendritic and pigmented melanocytes in one type of benign epidermal tumour. They felt that the melanocytes showed neoplastic rather than reactive features, and considered the lesion to represent a benign mixed tumour of both melanocytes and keratinocytes, which they called "Melanoacanthoma". Nowadays, however, most opinions consider that this is not a distinct entity but rather a variant of basal cell papilloma (Lever, 1975 and Bleehen, 1975) in which there is a block of pigment transfer from melanocyte to keratinocyte.

The situation in the unusual lesion described here is apparently very similar to that seen in the "melanoacanthoma" variant of basal cell papilloma, except that the tumour cells were derived from the epithelium of duct or ductular elements of breast, rather than epidermis. The melanocytes showed both hypertrophy and hyperplasia, and had migrated from their normal situation in the base of the epidermis. Ultrastructurally, they are best considered as hyperactive, rather than neoplastic melanocytes. Although their melanosomal morphology was similar to that seen in lentigo maligna melanoma, their

nuclei and nucleoli did not exhibit the malignant characteristics which are seen in this tumour (see page 185 and Plate 60).

One can only speculate on the mechanisms involved. It is possible that the oncogenic agent responsible for the breast carcinoma also caused hypertrophy and hyperplasia of the melanocytes ("field change"). However it seems more likely, particularly in view of the intimate association between the two types of cell, that the tumour cells produced some substance that attracted the melanocytes and caused their hyperactivity (increased melanosome production and arborisation of dendritic processes). The overload of melanosomes in the melanocytes could also have been due in part to a block in their transfer to the tumour cells.

In conclusion it seems reasonable to suppose that, under certain conditions, epithelial cells can influence the activity of melanocytes, and that the profound arborisation of melanocytic dendrites noted in the case above, after ultraviolet irradiation and in conditions associated with post inflammatory pigmentation (Pinkus et al., 1959) is due to some factor secreted by the epithelial cell. The unusual case reported here therefore exemplifies the close relationship between melanocytes and cells of epithelial origin.

Chapter X

GENERAL DISCUSSION

Including

1. INTRODUCTION

2. ULTRAVIOLET IRRADIATION

3. SUCTION AND FRICTION STUDIES

4. HISTIOCYTOSIS X, MALIGNANT MELANOMA AND "PSEUDO-MELANOMA"

1. INTRODUCTION

Space has already been given to discussion of this work at the end of each chapter. In this section an attempt will be made to summarise and correlate the views so that an overall impression of the structure and function of dendritic cells, subjected to various forms of stress, is obtained.

2. ULTRAVIOLET IRRADIATION

There is now considerable knowledge of the processes involved in melanin pigmentation of human skin exposed to ultraviolet radiation. It has been summarised well by Pathak et al., (1971). There is little disagreement that ultraviolet irradiation effects primarily the melanocyte and surrounding keratinocytes of the epidermal melanin unit.

As soon as five minutes after ultraviolet exposure there is some darkening of the skin. This immediate pigment-darkening reaction has, until now, not been considered in this thesis. It was not a factor in the experiments described in Chapter IV, which are concerned with much later events in the

response to ultraviolet radiation. Most caucasoids who have sunbathed must be aware of its existence; it is the "false tan" which appears within a few minutes of sun exposure. It is probably due to the migration and redistribution of already existing melanosomes from basal to more superficial keratinocytes, and to an oxidation reaction occurring in the melanosomes which may convert them temporarily to more dense structures (Pathak et al., 1971). With cessation of ultraviolet exposure the immediate pigment-darkening reaction fades rapidly for the first ten to fifteen minutes, and then more gradually over the next four or five hours.

The more delayed process of <u>new</u> pigment formation (melanogenesis) begins at about 24 hours (see Chapter IV). It depends on an increase in number of functional melanocytes, an increase in tyrosinase activity and melanosome production, and an increase in transfer of new melanosomes to the surrounding keratinocytes in the epidermal melanin unit. Each step is probably under genetic control (page 30).

Proliferation of melanocytes or activation of 'dormant' dopa-negative melanocytes in response to ultraviolet irradiation has been demonstrated

conclusively by light microscopy (e.g. Pathak et al., 1965; Quevedo et al., 1965; Szabo, 1967 and Wolff and Winkelmann, 1967) and electron microscopy (Mishima, 1967; Zelickson and Mottaz, 1970).

An increase in tyrosinase activity in response to ultraviolet irradiation was demonstrated at the light microscopic level by Fitzpatrick et al., 1950. The experiments described in Chapter IV were the first to outline the subcellular events which occur in the ultraviolet-irradiated melanocyte. In the discussion section of Chapter IV it is argued that the results support the prime role of tyrosinase, rather than peroxidase, in the process of melanogenesis. As Toda and Fitzpatrick (1971) have shown that glutaraldehyde prefixation in such electron microscopic cytochemical experiments almost abolishes tyrosinase activity in melanosomes of later development than Stage II, the experiments described in Chapter IV cannot be expected to detect tyrosinase in the late stages of its transport from ribosome (origin) to melanosome. This is illustrated diagramatically in Fig. 16. In this, tyrosinase is represented as an X and the box encloses that part of its journey from ribosomes attached to the endoplasmic reticulum

(top right) to melanosomes (bottom) which can be followed using the ultrastructural dopa or tyrosine reactions described in Chapters IV and VIII.

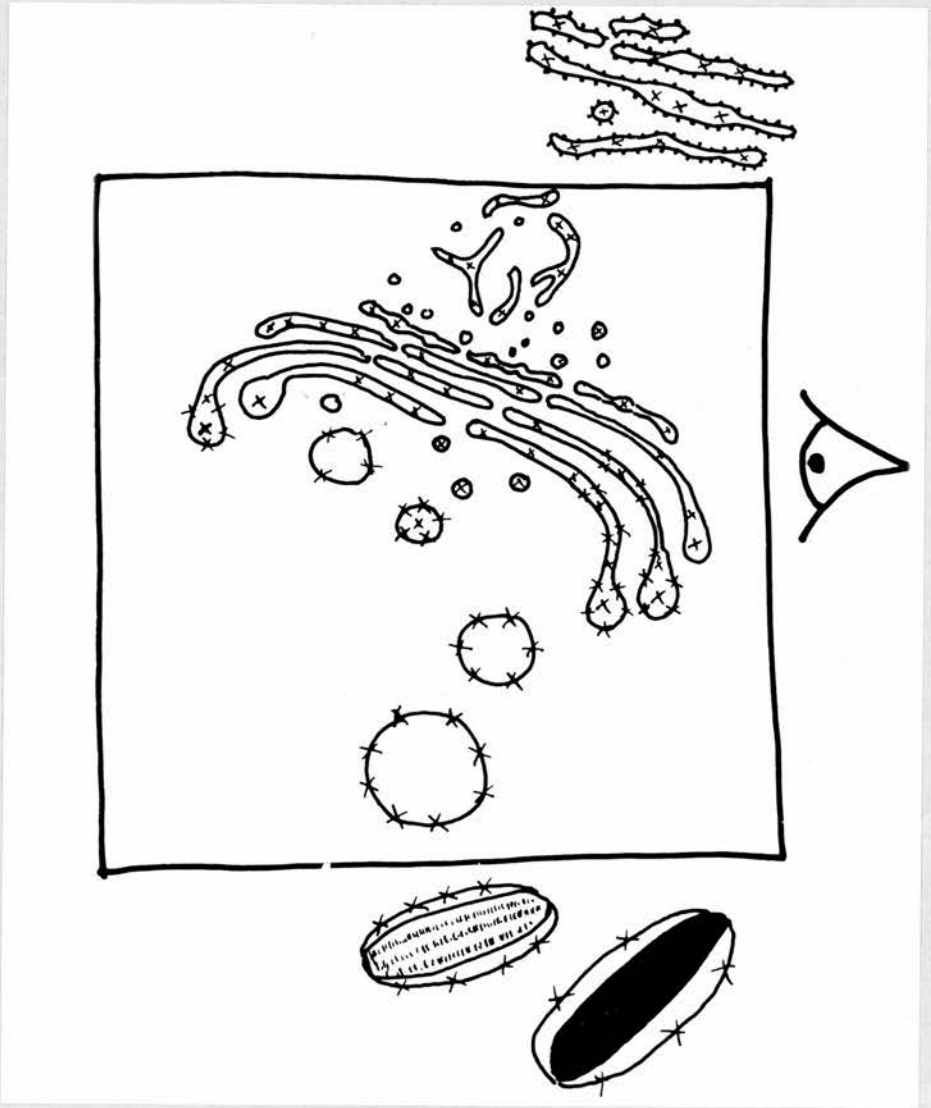


Figure 16: Diagram showing, in the box, localisation of tyrosinase (X) that can be detected using an ultrastructural dopa/tyrosinase reaction with glutaraldehyde prefixation.

The ultrastructural cytochemical experiments in Chapters IV and VIII support the view that tyrosinase passes from the rough endoplasmic reticulum to the Golgi apparatus via a tubular system of membranes (GERL), and is channelled from both the Golgi apparatus and GERL to focal dilatations of the smooth endoplasmic reticulum in which the earliest melanosomal structure has formed independently. Melanisation of the structural protein can then take place, and once this is complete the connection with the tubular system is severed. No major difference in the subcellular localisation of tyrosinase was noted between the ultraviolet-stimulated (Chapter IV) and neoplastic (Chapter VIII) melanocyte, though in some malignant cells there is an obvious arrest in development of the Stage I melanosome, so that large tyrosinase-positive vacuolar melanosomes are formed (Plates 79 and 80).

The precise way in which ultraviolet irradiation induces tyrosinase activity is not known, but the concept that its action is mediated by inhibition of glutathione reductase in the epidermis is intriguing, and seems sound (see page 19).

The increased tyrosinase activity in melanocytes after ultraviolet stimulation is also associated with an increase in the rate of melanosomal formation. When compared with non-irradiated skin, the irradiated skin of both caucasoids and negroids shows more melanosomes

in both melanocytes and keratinocytes (Szabo, 1967 and Szabo et al., 1969).

Less is known about the effects of ultraviolet irradiation on the transfer of melanosomes to surrounding keratinocytes of the epidermal melanin unit. However, considerable arborization of melanocyte dendrites is appreciable in biopsies obtained after ultraviolet irradiation (Pinkus et al., 1959), so this must represent one mechanism. It is also significant that defects at this stage (i.e. in the transfer of melanosomes from melanocytes to keratinocytes) have also been noticed in human skin exposed to prolonged solar radiation (Mitchell, 1963).

In Chapter IV the effect of ultraviolet irradiation on the Langerhans cell population was discussed in detail. At present it seems probable that there is no direct relationship, and that any change in the numbers of Langerhans cells following ultraviolet stimulation can be explained more logically on the basis of either mutual competition with melanocytes for territory in the basal cell layer (Breathnach, 1968 and Riley, 1975) or as a secondary effect of ultraviolet-induced changes in keratinocytes (Nix et al., 1964).

3. SUCTION AND FRICTION STUDIES

These studies exemplify certain physical differences between the keratinocyte and dendritic cell population. Melanocytes and Langerhans cells are hardly effected by either type of stress, yet there are profound changes in the general epidermal structure. It would seem that dendritic cells are able to withstand such insults because of their lack of attachment to neighbouring cells, and that this malleability provides an effective shock absorber system. The suction experiments also indicate that communications between the extracellular space and the system of endoplasmic reticulum, which have been demonstrated in keratinocytes (Hönigsmann and Wolff, 1973), do not exist in dendritic cells.

4. HISTIOCYTOSIS X, MALIGNANT MELANOMA AND "PSEUDO-MELANOMA"

These conditions can conveniently be discussed together. All represent proliferations of one type of dendritic cell population, and all are at present of unknown aetiology. Viral causes for both histiocytosis X (Nezelof and Basset, 1973) and malignant melanoma (Birkmayer et al., 1974) have been considered, but conclusive evidence is still lacking.

The ultrastructural findings in the single case of histiocytosis X reported here are similar to those recorded by others. Frequent cells in the histiocytic

infiltrate contain granules that are identical with those seen in the normal epidermal Langerhans cell. The concept that histiocytosis X is a proliferative disorder of pathological epidermal Langerhans cells (see Discussion Chapter VII) is most attractive and is supported by the findings reported here. However, even if this is accepted, it must be questioned whether it necessarily indicates a histiocytic role for the normal Langerhans cell. Nezelof and Basset (1973) have argued that there are more common features than differences between histiocytosis X cells and histiocytes (see page 163), and therefore consider that the histiocytosis X cell represents "a proliferative disorder of a highly differentiated and specialized cell: the Langerhans histiocyte." Although they deduce that the normal epidermal Langerhans cell has a histiocytic function, other experiments (see page 54) have failed to confirm a major phagocytic potential.

Unfortunately the experiments in this thesis do not shed much further light on the function of this mysterious cell. However, considering the experimental results reported here and recent publications relating to the function of the Langerhans cell, it would seem (almost by exclusion) that two possible avenues warrant further exploration. Studies which investigate

the role of the Langerhans cell in cell mediated reactions (see page 53) should be worthwhile, and it would be interesting to learn more about the function of the cell in relationship to epidermal turnover time (see page 56).

The picture in histiocytosis X is essentially an orderly one and is best considered as a well controlled but exaggerated response to an unknown stimulus. On structural grounds it is similar to the type of response seen in the proliferative melanocytes of lentigo maligna melanoma (Chapter VIII) and "pseudo-melanoma" (Chapter IX). It seems totally different from the picture seen in superficial spreading and nodular melanomas where order is replaced by anarchy, and the melanosomes can hardly be recognised as such.

That histiocytosis X and lentigo maligna melanoma are malignant processes cannot be denied, as both conditions can be rapidly invasive and destructive, and both may metastasize. Nevertheless the clinical course and response to treatment in these conditions suggests that the cellular proliferation is not so uncontrolled that it cannot be influenced by therapeutic means. Both can be considered as proliferative disorders of highly differentiated and specialized cells with consequent low malignancy.

In superficial spreading and nodular malignant melanoma the situation is very different. The studies

in Chapter VIII support Mishima's original view (1967) that there are differences between the oncogenesis of lentigo maligna melanoma and other types of malignant melanoma. However Mishima (Mishima and Matsunaka, 1975) believes that these differences depend on whether the malignant cells develop from preceding naevus cells (naevocytic melanoma) or melanocytes (melanocytic melanoma) (Fig. 17).

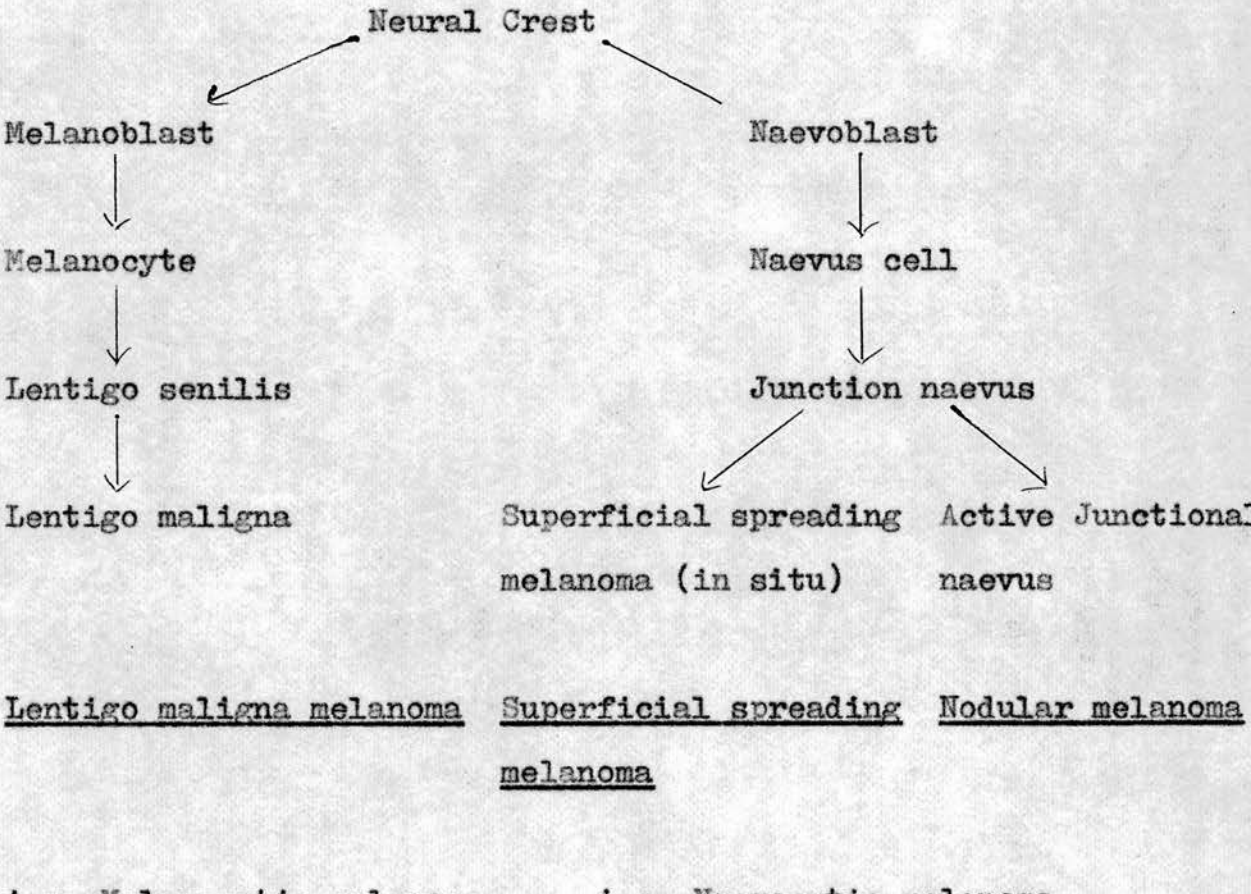


Figure 17: Simplified diagram of Mishima's views on oncogenesis of malignant melanoma.
(From Mishima and Matsunaka, 1975)

Mishima's concept is undoubtedly attractive, but our work and that of others (see discussion in Chapter VIII) show that there is scant structural evidence to support the idea of a naevus cell origin for all melanomas other than the lentigo maligna type.

The results of the experiments described in Chapter VIII also indicate an overlap in the ultrastructural appearance of lentigo maligna melanoma when compared with the other types, as noted by Clark et al., (1972). For example, in a few cases of lentigo maligna melanoma, the fine structure was more in keeping with that of superficial spreading or nodular melanoma and vice versa. Some might consider that this supports the concept of a spectrum of malignant dedifferentiation, rather than two sharply defined histogenetic or oncogenetic categories, as suggested on page 222.

Perhaps a more interesting facet of the study will become apparent in a few years time. It would be both intriguing and helpful to know whether prognosis can be related to ultrastructural appearance, as this would have obvious therapeutic implications (see page 214).

The dopa studies in Chapter VIII provided some

interesting information. The variability of dopa positivity of tumour cells was obvious, some showing gross deposition of reaction product whilst neighbouring cells showed none. This suggests a possible drawback in any therapeutic or diagnostic procedure which might depend on dopa uptake by malignant cells. The dopa studies also indicated that some of the undifferentiated cells (Type IV - Clark et al., 1972) were probably young tumour cells in which melanogenesis is just beginning, but in which melanosomes have not yet been formed.

The 'pseudo-melanoma' described in Chapter IX is basically a curiosity. The melanocytes in it were highly active and, on ultrastructural grounds, similar to those of lentigo maligna. The unusual picture merely serves as a reminder of the close relationship between epithelial cells and melanocytes. Either an unknown oncogenic agent caused proliferation of both breast epithelial cells and local melanocytes (field change), or melanocyte activity was induced by a product of the breast carcinoma.

Finally the tumour studies presented here provide little support for a close relationship between the Langerhans cell and the melanocyte. In histiocytosis X the melanocytes appeared unremarkable, and the Langerhans cells were normal in malignant melanoma.

REFERENCES

ABELE, D.C., GRIFFIN, T.B. (1972) Histiocytic medullary reticulosis. Archives of Dermatology, 106, 319.

ANTON-LAMPRECHT, I., SCHNYDER, U.W., TILGEN, W. (1971) Das "Stade éphélide" der melanotischen präcancerose. Archiv für Dermatologische Forschung, 240, 61.

ANTON-LAMPRECHT, I., TILGEN, W. (1972) Zur ultrastruktur der melanotischen präcancerose. Archiv für Dermatologische Forschung, 244, 264.

ARAO, T., KUWUHARA, H., INOUE, S. (1971) Clinicopathological and electronmicroscopical studies on melanotic freckle Hutchison and malignant melanoma arising from the melanotic freckle. Japanese Journal of Dermatology (Series B), 81, 444.

BAINTON, D.F., FARQUHAR, M.G. (1966) Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. Journal of Cell Biology, 28, 277.

BASSET, F., NEZELOF, C. (1969) L'histiocytose X. Microscopie électronique. Culture "in vitro" et histo-enzymologie. Discussion a propos de 21 cas. Revue Francais d'études Cliniques et Biologiques, XIV, 31.

BASSET, F., NEZELOF, C., MALLET, R., TURIAF, J. (1965) Nouvelle mise en évidence, par la microscopie électronique, de particules d'allure virale dans une seconde forme clinique de l'histiocytose X, le granulome éosinophile de l'os. Compté rendu Hebdomodaire des Séances de L'Académie des Sciences, 261, 5719.

BECKER, S.W. (1927) A systematic study of the pigment of human skin and the upper mucous membranes with special consideration of pigmented dendritic cells. Archives of Dermatology and Syphilology, 16, 259.

BECKER, S.W., PRAVER, L.L., THATCHER, H. (1935) An improved (paraffin section) method for the dopa reaction with considerations of the dopa-positive cell, as studied by this method. Archives of Dermatology and Syphilology, 31, 190.

BEHNKE, O. (1968) Electron microscopic observations on the surface coating of human blood platelets. Journal of Ultrastructural Research, 24, 51.

BILLINGHAM, R.E. (1948) Dendritic cells. Journal of Anatomy, 82, 93.

BILLINGHAM, R.E. (1949) Dendritic cells in pigmented human skin. Journal of Anatomy, 83, 109.

BILLINGHAM, R.E., MEDAWAR, P.B. (1953) A study of the branched cells of the mammalian epidermis with special reference to the fate of their division products. Philosophical Transactions of the Royal Society. Series B. 237, 151.

BIRBECK, M.S.C. (1962) Electron microscopy of melanocytes. British Medical Bulletin, 18, 220.

BIRBECK, M.S., BREATHNACH, A.S., EVERALL, J.D. (1961) An electron microscope study of basal melanocytes and high-level clear cells (Langerhans cells) in vitiligo. Journal of Investigative Dermatology, 37, 51.

BIRBECK, M.S.C., MERCER, E.H., BARNICOT, N.A. (1956) The structure and formation of pigment granules in human hair. Experimental Cell Research, 10, 505.

BIRKMAYER, G.D., BALDA, B.R., MILLER, F. (1974) Oncoma-viral information in human melanoma. European Journal of Cancer, 10, 419.

BLEEHEN, S.S. (1975) Pigmented basal cell epithelioma. Light and electron microscopic studies on tumours and cell cultures. British Journal of Dermatology, 93, 361.

BLEEHEN, S.S., PATHAK, M.A., HORI, Y., FITZPATRICK, T.B. (1968) Depigmentation of skin with 4-isopropylcatechol, mercaptoamines, and other compounds. Journal of Investigative Dermatology, 50, 103.

BLOCH, B. (1917) Chemische untersuchungen über das spezifische pigmentbildende ferment der haut, die dopa oxidase. Zeitschrift fur Physiologische Chemie, XCViii, 226.

BOUISSON, H., DURROUX, R., FABRE, J., REGNIER, C. (1967) Etude ultrastructurale de l'histiocytose X. Compté rendu Hebdomodaire des Séances de L'Académie des Sciences, 265D, 1266.

BOURQUELOT, E., BERTRAND, G. (1895) Le blenissement et le noircissement des champignons. Comptés Rendus des Séances de la Societé de Biologie, 47, 582.

BREATHNACH, A.S. (1963a) A new concept of the relation between the Langerhans cell and the melanocyte. Journal of Investigative Dermatology, 40, 279.

BREATHNACH, A.S. (1963b) The distribution of Langerhans cells within the human hair follicle and some observations on its staining properties with gold chloride. Journal of Anatomy, 97, 73.

BREATHNACH, A.S. (1964) Observations on cytoplasmic organelles in Langerhans cells of human epidermis. Journal of Anatomy, 98, 265.

BREATHNACH, A.S. (1965) The cells of Langerhans. International Review of Cytology, 18, 1.

BREATHNACH, A.S. (1971) An Atlas of the Ultrastructure of Human Skin. Churchill, London.

BREATHNACH, A.S., BIRBECK, M.S., EVERALL, J.D. (1962) Observations on Langerhans cells in leprosy. British Journal of Dermatology, 74, 243.

BREATHNACH, A.S., BIRBECK, M.S.C., EVERALL, J.D. (1963) Observations bearing on the relationship between Langerhans cells and melanocytes. Annals of the New York Academy of Science, 100, 223.

BREATHNACH, A.S., FITZPATRICK, T.B., WYLLIE, L.M.A. (1965) Electron microscopy of melanocytes in human piebaldism. Journal of Investigative Dermatology, 45, 28.

BREATHNACH, A.S., GOODWIN, D.P. (1965) Electron microscopy of non-keratinocytes in the basal layer of white epidermis of the recessively spotted guinea-pig. Journal of Anatomy, 99, 377.

BREATHNACH, A.S., SILVERS, W.K., SMITH, J., HEYNER, S. (1968) Langerhans cells in mouse skin experimentally deprived of its neural crest component. Journal of Investigative Dermatology, 50, 147.

BREATHNACH, A.S., WYLLIE, L.M. (1965a) Electron microscopy of melanocytes and Langerhans cells in human fetal epidermis at fourteen weeks. Journal of Investigative Dermatology, 44, 51.

BREATHNACH, A.S., WYLLIE, L.M. (1965b) Melanin in Langerhans cells. Journal of Investigative Dermatology, 45, 401.

BROWN, J., WINKELMANN, R.K., WOLFF, K. (1967) Langerhans cells in vitiligo: a quantitative study. Journal of Investigative Dermatology, 49, 387.

BRUMBAUGH, J., BOWERS, R., LEE, K. (1973) Histochemical evidence that peroxidase does not affect melanin formation in feather melanocyte. Yale Journal of Biology and Medicine, 46, 523.

BRUMBAUGH, J.A., ZIEG, R.H. (1972) The ultrastructural effects of the dopa reaction upon developing retinal and epidermal melanocytes in the fowl: in Pigmentation. Its Genesis and Biologic Control. Ed. V. Riley, Appleton Century Crofts, New York. p.107

BURNETT, J.B., SEILER, H., BROWN, I.V. (1967) Separation and characterization of multiple forms of tyrosinase from mouse melanoma. Cancer Research, 27, 880.

CANCILLA, P.A., LAHEY, M.E., CAMES, W.H. (1967) Cutaneous lesions of Letterer Siwe disease. Electron microscopy study. Cancer, 20, 1986.

CARO, L.G., PALADE, G.E. (1964) Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study. Journal of Cell Biology, 20, 473.

CARPENTER, A.M., LAZAROW, A. (1962) Component quantitation of tissue sections. Journal of Histochemistry and Cytochemistry, 10, 329.

CAUDIERE, M. (1926) Recherches sur l'evolution des cellules pigmentaires dans certains epitheliomas envahissant l'epiderme. Annales d'Anatomie Pathologique, 3, 119.

CAUNA, N.,MACY, I.L., CRALLEY, A. (1970) The fine morphology of the human skin in dermographism. Journal of Allergy, 45, 266.

CESARINI, J.P. (1971) Recent advances in the ultrastructure of malignant melanoma. Revue Europeene d'études Cliniques et Biologiques, 16, 316.

CHANG, S.C. (1972) Haematoxylin-eosin staining of plastic-embedded tissue sections. Archives of Pathology, 93, 344.

CHARLES, A., INGRAM, J.T. (1959) Electron microscope observations of the melanocyte of the human epidermis. Journal of Biophysical and Biochemical Cytology, 6, 41.

CHIAN, L.T., WILGRAM, G.F. (1967) Tyrosinase inhibition: its role in suntanning and in albinism. Science, 155, 198.

CHRISTOPHERS, E., LAWRENCE, E.G. (1973) Regional variations in mouse skin: quantitation of epidermal compartments in two different body sites. Virchows Archiv Abteilung B Zellpathologie, 12, 212.

CLARK, R.L., MARGULIES, S.I., MULHOLLAND, J.H. (1970) Histiocytosis X: a fatal case with unusual pulmonary manifestations. Radiology, 95, 631.

CLARK, W.H. (1967) A classification of malignant melanoma in man correlated with histogenesis and biologic behaviour: in Advances in Biology of Skin Vol. VIII. The Pigmentary System. Ed. W. Montagna and F. Hu, Pergamon Press, Oxford. p.621.

CLARK, W.H., BRETTON, R. (1972) A comparative fine structural study of melanogenesis in normal human epidermal melanocytes and in certain human malignant melanoma cells: in The Skin (International Academy of Pathology Monograph). Ed. E.B. Helwig, Williams and Wilkins & Co., Baltimore. p.197.

CLARK, W.H. Jr.,FROM, L., BERNADINO, F.A., MIHM, M.C. (1969) The histogenesis and biologic behaviour of primary human malignant melanoma of skin. Cancer, 29, 705.

CLARK, W.H. Jr., HEGGELER, B., BRETTON, R. (1972) Electron microscopic observations of human cutaneous melanomas correlated with their biologic behaviour: in Melanoma and Skin Cancer. Ed. W.H. McCarthy, Government Printer, V.C.N. Blight, Sydney. p.121.

COMAISH, J.S. (1973) Glutaraldehyde lowers skin friction and enhances skin resistance to acute friction injury. Acta Dermato-venereologica, 53, 455.

CONSTABLE, H. (1975) The structure and behaviour of epidermal cells in tissue culture. University of Birmingham, Ph.D. Thesis.

COPEMAN, P.W.M. (1970) Porphyria: successful treatment by alkalinization of urine with sodium bicarbonate assessed by experimental suction blister apparatus. British Journal of Dermatology, 82, 385.

COPEMAN, P.W.M., BLEEHEN, S.S., LEWIS, M.G. (1973) Biology and immunology of vitiligo and malignant melanoma: in Recent Advances in Dermatology. Ed. A.J. Rook, Churchill Livingstone, Edinburgh. p.245.

CRUICKSHANK, C.N.D., COOPER, J.R. (1975) Langerhans cells in tissue cultures of guinea-pig epidermal cells. British Journal of Dermatology, 92, 121.

CRUICKSHANK, C.N.D., HARCOURT, S.A. (1964) Pigment donation in vitro. Journal of Investigative Dermatology, 42, 183.

CUBIE, H.A. (1972) Laboratory investigations in the study of human papilloma virus. M.Sc. thesis, University of Edinburgh.

DE MAN, J.C.H. (1968) Rod like tubular structures in the cytoplasm of histiocytes in "Histiocytosis X". Journal of Pathology and Bacteriology, 95, 123.

DOGGENWEILER, C.F., FRENK, S. (1965) Staining properties of lanthanum on cell membranes. Proceedings of the National Academy of Sciences of the United States of America, 53, 425.

DROCHMANS, P. (1960) Electron microscopic studies of epidermal melanocytes and the fine structure of melanin granules. Journal of Biophysical and Biochemical Cytology, 8, 165.

DROCHMANS, P. (1967) Ultrastructure of melanin granules: in Advances in Biology of Skin viii. The Pigmentary System. Ed. W. Montagna and F. Hu, Pergamon Press, Oxford. p.169

DRURY, R.A.B., WALLINGTON (1967) Carleton's Histological Technique, 4th Edition. Oxford University Press, Oxford.

DUBUY, H.G., SHOWACRE, J.L., HESSELBACH, M.L. (1963) Enzymatic and other similarities of melanoma granules and mitochondria: in The Pigment Cell: Molecular, Biological and Clinical Aspects. Ed. V. Riley and J.G. Fortner, New York Academy of Sciences, 100, 569.

DUCHON, J., FITZPATRICK, B., SEIJI, M. (1968) Melanin 1968. Some definitions and problems: in Year Book of Dermatology, 1967/68 series. Ed. A.W. Kopf and Rafael Andrade, Year Book Medical Publishers, Chicago. p.6.

EBLING, F.J. (1974) Personal communication.

EPSTEIN, W.L., FUKUYAMA, K., CORTESE, T.A. (1969) Autoradiographic study of friction blisters, RNA, DNA and protein synthesis. Archives of Dermatology, 99, 94.

FAN, J., SCHOENFIELD, R.J., HUNTER, R. (1959) A study of the epidermal clear cells with special reference to their relationship to the cells of Langerhans. Journal of Investigative Dermatology, 32, 445.

FARQUHAR, M.G., PALADE, G.E. (1965) Cell junctions in amphibian skin. Journal of Cell Biology, 26, 263.

FERREIRA-MARQUES, J. (1951) Systema sensitivum intraepidermiscum. Die Langerhanschen zellen orls rezeptoren des hellen schmerzes: Doliriceptores. Archiv für Dermatologie und Syphilis, 193, 191.

FITZPATRICK, T.B., BECKER, S.W. Jr., LERNER, A.B. MONTGOMERY, H. (1950) Tyrosinase in human skin: demonstration of its presence and of its role in human melanin formation. Science, 112, 223.

FITZPATRICK, T.B., BREATHNACH, A.S. (1963) Das epidermale melanin-einheit-system. Dermatologische Wochenschrift, 147, 481.

FITZPATRICK, T.B., LERNER, A.B. (1953) Terminology of pigment cells. Science, 117, 640.

FITZPATRICK, T.B. MIYAMOTO, M., ISHIKAWA, K. (1967) The evolution of concepts of melanin biology. Archives of Dermatology, 96, 305.

FITZPATRICK, T.B., QUEVEDO, W.C. Jr., SZABO, G., SEIJI, M. (1971) Biology of the melanin pigmentary system: in Dermatology in General Medicine. Ed. T.B. Fitzpatrick, K.A. Arndt, W.H. Clark, A.Z. Eisen, E.J. Van Scott and J.H. Vaughan, McGraw Hill, New York. p.117.

FRITSCH, P. (1971) Langerhans zellen in viruswargen unter suchungen zur frage von beziehungen zwischen Langerhans zellen und verhornung. Archiv für Dermatologische Forschung, 242, 70.

FROST, P., WEINSTEIN, G.D., VAN SCOTT, E.J. (1966) The ichthyosiform dermatosis, II. Autoradiographic studies of epidermal proliferation. Journal of Investigative Dermatology, 47, 561.

FUKUYAMA, K., CORTESE, T.A. (1968) Experimental friction blisters: histological investigation: in Proceedings of the 13th International Congress of Dermatology. Ed. W. Jadassohn and C.G. Schirren, Springer, Berlin. p.1288.

GIACOMETTI, L. (1968) Cells in motion. Primate news. Medical Research Foundation, Oregon. p.3.

GIACOMETTI, L. (1969) The healing of skin wounds in primates III. Behaviour of the cells of Langerhans. Journal of Investigative Dermatology, 53, 151.

GIACOMETTI, L., BARSS, M. (1969) Paul Langerhans: a tribute. Archives of Dermatology, 100, 770.

GIANOTTI, F., CAPUTO, R., RANZI, T. (1968) Ultrastructural study of giant cells and Langerhans cell granules in cutaneous lesions and lymph node and liver biopsies from four cases of subacute disseminated histiocytosis of Letterer-Siwe. Archiv für Klinische und Experimentelle Dermatologie, 233, 238.

GIACOMETTI, L., MONTAGNA, W. (1967) Langerhans cells: uptake of tritiated thymidine. Science, 157, 439.

GLAUERT, A.M., GLAUERT, R.H. (1958) Araldite as an embedding medium for electron microscopy. Journal of Biophysical and Biochemical Cytology, 4, 191.

HABESHAW, J.A., STUART, A.E. (1975) Cell receptor studies on seven cases of diffuse histiocytic malignant lymphoma (reticulum cell sarcoma). Journal of Clinical Pathology, 28, 289.

HACKMANN, M., GRUBB, C., HILL, K.R. (1968) The ultrastructure of normal squamous epithelium of the human cervix uteri. Journal of Ultrastructural Research, 22, 443.

HAELST, U.J.C. Van (1969) Light and electron microscopic studies of the normal and pathological thymus of the rat III. A mesenchymal histiocytic type of cell. Zeitschift für Zellforschung und mikroskopische Anatomie, 99, 198.

HALPRIN, K.M. (1972) Epidermal "turnover time" - a re-examination. British Journal of Dermatology, 86, 14.

HALPRIN, K.M., OHKAWARA, A. (1967) Human pigmentation: the role of glutathione: in The Pigmentary System. Vol. 8 Advances in Biology of the Skin. Ed. W. Montagna and F. Hu, Pergamon Press, New York.p.241.

HASHIMOTO, K. (1970) Lanthanum staining of Langerhans cell. Archives of Dermatology, 102, 280.

HASHIMOTO, K. (1971) Langerhans cell granule. An endocytic organelle. Archives of Dermatology, 104, 148.

HASHIMOTO, K., TARKOWSKI, W.M. (1968) Some new aspects of the Langerhans cell. Archives of Dermatology, 97, 450.

HENDERSON, D.W., SAGE, R.E. (1973) Malignant histiocytosis with eosinophilia. Cancer, 32, 1421.

HIRONE, T., NAGAI, T., MATSUBARA, T., FUKUSHIRO, T. (1971) Human malignant melanomas of the skin and their pre-existing conditions: in Biology of Normal and Abnormal Melanocytes. Ed. T. Kawamura, T.B. Fitzpatrick and M. Seiji, University Park Press, Baltimore. p.329.

HOLSTEIN, T.J., STOWELL, C.P., QUEVEDO, W.C., ZARCARO, R.M., BIENIEKI, T.C. (1973) Peroxidase, "protyrosinase", and the multiple forms of tyrosinase in mice. Yale Journal of Biology and Medicine, 46, 560.

HOLTZMAN, E., DOMINITZ, R. (1968) Lysosomes, Golgi apparatus and endoplasmic reticulum in secretion and protein uptake by adrenal medulla cells of the rat. Journal of Histochemistry and Cytochemistry, 16, 320.

HÖNIGSMANN, H., WOLFF, K. (1973) Continuity of intercellular space and endoplasmic reticulum of keratinocytes. Journal of Experimental Cell Research, 80, 191.

HORI, Y., TODA, K., PATHAK, H.A., CLARK, W.H. Jr., FITZPATRICK, T.B. (1968) A fine structure study of the human epidermal melanosome complex and its acid phosphatase activity. Journal of Ultrastructural Research, 25, 109.

HUNTER, J.A.A. (1972a) Electron microscopy - a functional approach. British Journal of Dermatology, 87, 53.

HUNTER, J.A.A. (1972b) Current concepts on the role of the Langerhans cell. Scottish Medical Journal, 17, 232.

HUNTER, J.A.A., FAIRLEY, D.J., PRIESTLEY, G.C., CUBIE, H.A. (1976) Langerhans cells in the epidermis of athymic mice. British Journal of Dermatology, 94, 119.

HUNTER, J.A.A., MCVITTIE, E., COMAISH, J.S. (1974a) Light and electron microscopic studies of physical injury to the skin. I suction. British Journal of Dermatology, 90, 481.

HUNTER, J.A.A., MCVITTIE, E., COMAISH, J.S. (1974b) Light and electron microscopic studies of physical injury to the skin. II friction. British Journal of Dermatology, 90, 491.

HUNTER, J.A.A., MOTTAZ, J.H., ZELICKSON, A.S. (1970) Melanogenesis: ultrastructural histochemical observations on ultraviolet irradiated human melanocytes. Journal of Investigative Dermatology, 54, 213.

HUTCHENS, L.H., SAGEBIEL, R.W., CLARKE, M.A. (1971) Oral epithelial dendritic cells of the rhesus monkey. Histologic demonstration, fine structure and quantitative distribution. Journal of Investigative Dermatology, 56, 325.

HUVOS, A.G., MIKÉ, V., DONNELLAN, M.J., SEEMAYER, T., STRONG, E.W. (1973) Prognostic factors in cutaneous melanoma of the head and neck. American Journal of Pathology, 71, 33.

INAMURA, M., SAKAMOTO, S., HANAZONO, H. (1971) Malignant histiocytosis: a case of generalised histiocytosis with infiltration of Langerhans granule containing histiocytes. Cancer, 28, 467.

ISHIBASHI, A., KASUGA, T., TSUCHIYA, E. (1971) Electron microscopy of basal cell carcinoma. Journal of Investigative Dermatology, 56, 298.

JAKUBOWICZ, K., DABROWSKI, J., BICZYSKO, W., WALSKI, M. Ultrastruktur der melanosomen in melanomalignom. Dermatologi Monatsschrift, 156, 299

JAMIESON, J.D., PALADE, G.E. (1967) Intracellular transport of secretory proteins in the pancreatic exocrine cell. II Transport to condensing vacuoles and zymogen granules. Journal of Cell Biology, 34, 597.

JIMBOW, K., SATO, S., KUKITA, A. (1969a) Langerhans cells of the normal human pilosebaceous system. An electron microscopic investigation. Journal of Investigative Dermatology, 52, 177.

JIMBOW, K., SATO, S., KUKITA, A. (1969b) Cells containing Langerhans granules in human lymph nodes of dermatopathic lymphadenopathy. Journal of Investigative Dermatology, 53, 295.

JOURDAIN, J.C. (1974) Étude de critères cliniques et histologiques de prognostic des mélanomes malins primaires. Annales Dermatologie et Syphiligraphie, 101, 171.

KARNOVSKY, M.J. (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. Journal of Cell Biology, 27, 137A.

KIISTALA, U., MUSTAKALLIO, K.K. (1967) Dermo-epidermal separation with suction. Electron microscopic and histochemical study of initial events of blistering on human skin. Journal of Investigative Dermatology, 48, 466.

KLINGMÜLLER, G., REUTER, G., RODERMUND, O.E., SCHMOECKEL, Ch. (1970) Die submikroskopie des malignen melanoms. Dermatologische Monatsschrift, 156, 308.

KLUG, H., GÜNTHER, W. (1972) Ultrastructural differences in human malignant melanoma. An electron microscopic study. British Journal of Dermatology, 86, 395.

KONRAD, K., WOLFF, K. (1973) Hyperpigmentation, melanosome size, and distribution patterns of melanosomes. Archives of Dermatology, 107, 853.

KONRAD, K., WOLFF, K., HÖNIGSMANN, H. (1974) The giant melanosome: a model of deranged melanosome morphogenesis. Journal of Ultrastructural Research, 48, 102.

KOURI, J., ANCHETA, O. (1972) Transformation of macrophages into fibroblasts. Experimental Cell Research, 71, 168.

LANGERHANS, P. (1868) Uber die nerven der menschlichen haut. Virchows Archiv für pathologische Anatomie und Physiologie und für Klinische Medizin, 44, 325.

LANGEVOORT, H.L., COHN, Z.A., HIRSCH, J.G., HUMPHREY, J.H., SPECTOR, W.G., VAN FURTH, R. (1970) The nomehclature of mononuclear phagocytic cells. Proposal for a new classification: in Mononuclear Phagocytes. Ed. R. Van Furth, Blackwell Scientific Publications, Oxford, p.1.

LERNER, A.B., FITZPATRICK, T.B. (1950) Biochemistry of melanin formation. Physiological Reviews, 30, 91.

LERNER, A.B., FITZPATRICK, T.B., CALKINS, E., SUMMERSON, W.H. (1949) Mammalian tyrosinase: preparation and properties. Journal of Biological Chemistry, 178, 185.

LESSARD, R., WOLFF, K., WINKELMANN, R.K. (1966) Induced 'shedding' of the epidermal Langerhans cells. Nature, 212, 628.

LESSARD, R.J., WOLFF, K., WINKELMANN, R.K. (1968) The disappearance and regeneration of Langerhans cells following epidermal injury. Journal of Investigative Dermatology, 50, 171.

LEVER, W.F., SCHAUMBERT-LEVER, G. (1975) Histopathology of the Skin, 5th Edition. J.B. Lippincott Co., Philadelphia.

LICHTENSTEIN, L. (1953) Histiocytosis X: integration of eosinophilic granuloma of bone, Letterer-Siwe disease and Schüller-Christian disease as related manifestations of a single nosologic entity. Archives of Pathology, 56, 84.

LITTLE, J.H. (1972) Histology and prognosis in cutaneous malignant melanoma: in Melanoma and Skin Cancer. Ed. W.H. McCarthy, Government Printer, V.C.N. Blight, Sydney. p.107.

LOWE, L.B., VAN DER LEUN, J.C. (1968) Suction blisters and dermal-epidermal adherance. Journal of Investigative Dermatology, 50, 308.

LUCE, J.K., MCBRIDE, C.M., FREI, E. (1973) Melanoma: in Cancer Medicine. Ed. J.F. Holland and E. Frei, Lea and Febiger, Philadelphia. p.1823.

LUFT, J.H. (1961) Improvements in epoxy resin embedding methods. Journal of Biophysical and Biochemical Cytology, 9, 409.

LUPULESCU, A., PINKUS, H., BIRMINGHAM, D.J., USNDEK, H.E., POSCH, J.L. (1973) Lentigo maligna of the fingertip. Clinical, histologic and ultrastructural studies of two cases. Archives of Dermatology, 107, 717.

MCGOVERN, V.J. (1970) The classification of melanoma and its relationship with prognosis. Pathology, 2, 85.

MCGOVERN, V.J., LANE BROWN, M.M. (1969) The Nature of Melanoma. Charles C. Thomas, Springfield, Illinois. p.100.

MCGOVERN, V.J., MIHM, M.C., BAILLY, C., BOOTH, J.C., CLARK, W.H., COCHRAN, A.J., HARDY, E.G., HICKS, J.D., LEVENE, A., LEWIS, M.G., LITTLE, J.H., MILTON, G.W. (1973) The classification of a malignant melanoma and its histologic reporting. Cancer, 32, 1446.

MACKENZIE, I.C. (1972) The ordered structure of mammalian skin: in Epidermal Wound Healing. Ed. H.I. Maibach and D. Rowe, Medical Year Book Publishers, Chicago.

MACKENZIE, I.C. (1974) The effects of oral administration of chlorhexidine on the rate of regeneration of epithelia of the oral mucosa and skin of mice. Journal of Periodontal Research, 9, 181.

MACKENZIE, I.C. (1975) Labelling of murine epidermal Langerhans cells with H^3-Thymidine. American Journal of Anatomy, 144, 127.

MACKENZIE, I.C., SQUIER, C.A. (1975) Cytochemical identification of A.T.P.'ase positive Langerhans cells in EDTA - separated sheets of mouse epidermis. British Journal of Dermatology, 92, 523.

MASON, H.S., KAHLER, H., MACCARDLE, R.C., DALTON, A.J. (1947) Chemistry of Melanin IV. Electron micrography of natural melanins. Proceedings of the Society for Experimental Biology and Medicine, 66, 421.

MASON, H.S., PETERSON, E.W. (1965) Melanoproteins 1: reactions between enzyme-generated quinones and amino acids. Biochimica et Biophysica Acta, 111, 134.

MASSON, P. (1926) Les naevi pigmentaires, tumeurs nerveuses. Annales d'Anatomie Pathologique, 3, 417

MASSON, P. (1948) Pigment cells in man: in The Biology of Melanosomes. Ed. R.W. Miner and M. Gordon, New York Academy of Science (special publication), IV, 15.

MASSON, P. (1951) My conception of cellular naevi. Cancer, 4, 9.

MAUL, G.G. (1969) Golgi-melanosome relationship in human melanoma in vitro. Journal of Ultrastructure Research, 26, 163.

MAUL, G.G., BRUMBAUGH, J. (1971) On the possible function of coated vesicles in melanogenesis of the regenerating fowl feather. Journal of Cell Biology, 48, 41.

MISHIMA, Y. (1960) Melanosis circumscripta praecancerosa (Dubreuilh) - a non-nevoid premelanoma distinct from junction naevus. Journal of Investigative Dermatology, 34, 361.

MISHIMA, Y. (1962) Electron microscopy of melanin synthesis in intradermal naevus cells. Journal of Investigative Dermatology, 39, 369.

MISHIMA, Y. (1965) Macromolecular changes in pigmentary disorders. Archives of Dermatology, 91, 519.

MISHIMA, Y. (1966) Melanosomes in phagocytic vacuoles in Langerhans cells. Electron microscopy of keratin-stripped human epidermis. Journal of Cell Biology, 30,417.

MISHIMA, Y. (1967) Melanocytic and nevocytic malignant melanomas: cellular and subcellular differentiation. Cancer, 20, 632.

MISHIMA, Y., KAWASAKI, H., PINKUS, H. (1972) Dendritic cell dynamics in progressive depigmentations. Archiv für Dermatologische Forschung, 243, 67.

MISHIMA, Y., MATSUNAKA, M. (1975) Pagetoid premalignant melanosis and melanoma: differentiation from Hutchinson's melanotic freckle. Journal of Investigative Dermatology, 65, 434.

MISHIMA, Y., MILLER-MILINSKA, A. (1961) Junctional and high-level dendritic cells revealed with osmium iodide reaction in human and animal epidermis under conditions of hyperpigmentation and depigmentation. Journal of Investigative Dermatology, 37, 107.

MISHIMA, Y., PINKUS, H. (1960) Benign mixed tumour of melanocytes and malpighian cells: melanoacanthoma. Archives of Dermatology, 81, 539.

MISHIMA. Y., WIDLAN, S. (1967) Enzymically active and inactive melanocyte populations and ultraviolet irradiation: combined dopa-premelanin reaction and electron microscopy. Journal of Investigative Dermatology, 49, 273.

MITCHELL, R.E. (1963) The effect of prolonged solar radiation on melanocytes of the human epidermis. Journal of Investigative Dermatology, 41, 199.

MOLLENHAUER, H.H., WHALEY, W.G. (1963) An observation on the functioning of the Golgi apparatus. Journal of Cell Biology, 17, 222.

MOLOKHIA, M.M., PORTNOY, B. (1971) A study of dendritic cells in seborrhoeic warts. British Journal of Dermatology, 85, 254.

MORALES, A.R., FINE, G., HORN, R.C., WATSON, J.H.L. (1969) Langerhans cells in a localized lesion of the eosinophilic granuloma type. Laboratory Investigation, 20, 412.

MOTTAZ, J.H., THORNE, E.G., ZELICKSON, A.S. (1971) Response of the epidermal melanocyte to minor trauma. Archives of Dermatology, 104, 611.

MOTTAZ, J.H., ZELICKSON, A.S. (1967) Melanin transfer: a possible phagocytic process. Journal of Investigative Dermatology, 49, 605.

MOYER, F.H. (1963) Genetic effects on melanosome fine structure and ontogeny in normal and malignant cells: in The Pigment Cell. Molecular, Biological and Clinical Aspects. Ed. M. Gordon, Annals of the New York Academy of Sciences, 100, 584.

MOYER, F.H. (1966) Genetic variations in the fine structure and ontogeny of mouse melanin granules. American Zoologist, 6, 43.

NAKAI, T., SHUBIK, P. (1964) Electron microscopic radioautography: the melanosome as a site of melanogenesis in neoplastic melanocytes. Journal of Investigative Dermatology, 43, 267.

NAYLOR, P.F.D. (1955) Experimental friction blisters. British Journal of Dermatology, 67, 327.

NEZELOF, C., BASSET, F., ROUSSEAU, M.F. (1973) Histiocytosis X. Histogenetic arguments for a Langerhans cell origin. Biomedicine, 18, 365.

NICOLAUS, R.A. (1962) Biogenesi delle melanine. Naples: Instituto di Chimica Organica dell'Universita di Napoli (as quoted by Duchon et al., 1968)

NICOLAUS, R.A., PIATELLI, M. (1965) Progress in the chemistry of natural black pigments. Rendiconti dell'Accademia delle Scienze Fisiche e Matematiche, 32, 1.

NIEBAUER, G., KRAWCZYK, W.S., KIDD, R.L., WILGRAM, G.F. (1969) Osmium zinc iodide reactive sites in the epidermal Langerhans cell. Journal of Cell Biology, 43, 80.

NIX, T.E. (1967) Ultraviolet-induced changes in epidermis: in Ultrastructure of Normal and Abnormal Skin. Ed. A.S. Zelickson, Lea and Febiger, Philadelphia. p. 304.

NIX, T.E. Jr., NORDQUIST, R.E., SCOTT, J.R., EVERETT, M.A. (1964) Ultrastructural changes in stratum corneum induced by ultraviolet light. Journal of Investigative Dermatology, 43, 301.

NIX, T.E. Jr., NORDQUIST, R.E., SCOTT, J.R., EVERETT, M.A. (1965) Ultrastructural epidermal changes induced by ultraviolet light: basal and spinous layers. Journal of Investigative Dermatology, 45, 52.

NORDQUIST, R.E., OLSON, R.L., EVERETT, M.A. (1966) The transport, uptake and storage of ferritin in human epidermis. Archives of Dermatology, 94, 482.

NOVIKOFF, A.B. (1967) Enzyme localization and ultrastructure of neurons and lysosomes in nerve cells: in The Neuron. Ed. H. Hyden, Elsevier Publishing Company, Amsterdam. p.255.

NOVIKOFF, A.B., ALBALA, A., BIEMPICA, L. (1968) Ultrastructural and cytochemical observations on B-16 and Harding-Passey mouse melanomas. The origin of premelanosomes and compound melanosomes. Journal of Histochemistry and Cytochemistry, 16, 299.

NOVIKOFF, A.B., ROHEIM, P.S., QUINTANA, N. (1966) Changes in rat liver cells induced by orotic acid feeding. Laboratory Investigation, 15, 27.

ODLAND, G.F. (1958) The fine structure of the interrelationship of cells in the human epidermis. Journal of Biophysical and Biochemical Cytology, 4, 529.

OKUN, M.R., EDELSTEIN, L.M., PATEL, R.P., DONNELLAN, B. (1973a) A revised concept of mammalian melanogenesis: the possible synergistic functions of aerobic dopa oxidase and peroxidase. A review. Yale Journal of Biology and Medicine, 46, 535.

OKUN, M.R., DONNELLAN, B., PATEL, R.P., LEON, M., EDELSTEIN, M.D. (1973b) Subcellular demonstration of peroxidatic oxidation of tyrosine to melanin using dihydroxyfumarate as cofactor in mouse melanoma cells. Journal of Investigative Dermatology, 61, 60.

PATHAK, M.A., HORI, Y., SZABO, G., FITZPATRICK, T.B. (1971) The photobiology of melanin pigmentation in human skin: in Biology of Normal andAbnormal Melanocytes. Ed. T. Kawamura, T.B. Fitzpatrick and M. Seiji, University Park Press, Baltimore. p.149.

PATHAK, M.A., SINESI, S.J., SZABO, G. (1965) The effect of a single dose of ultraviolet radiation on epidermal melanocytes. Journal of Investigative Dermatology, 45, 520.

PEARSON, R.W. (1965) Response of human epidermis to graded thermal stress: a morphologic comparison of burns, cold induced blisters, and pemphigus vulgaris. Archives of Environmental Health, 11, 498.

PEASE, D.C. (1951) Electron microscopy of human skin. Americal Journal of Anatomy, 89, 469.

PIEPER, J. (1973) Personal communication.

PINKUS, H., STARICCO, R.J., DROPP, P.J., FAN, J. (1959) The symbiosis of melanocytes and human epidermis under normal and abnormal conditions: in Pigment Cell Biology. Ed. M. Gordon, Academic Press, New York. p.127.

PLEWIG, G., WOLFF, H., BRAUN-FALCO, O. (1971) Lokalbehandlung normaler und pathologischer menschlicher haut mit vitamin a-saure, Klinische, histologische und elektronenmikroskopische Untersuchungen. Archiv fur Klinische und Experimentelle Dermatologie, 239, 390.

PROTA, G., NICOLAUS, R.A. (1967) On the biogenesis of phaeomelanins: in The Pigmentary System, Volume 8 of Advances in Biology of the Skin. Ed. W. Montagna and F. Hu, Pergamon Press, New York. p.323.

PRUNIERAS, M. (1969) Interactions between keratinocytes and dendritic cells. Journal of Investigative Dermatology, 52, 1.

QUEVEDO, W.C. (1971) Genetic regulation of pigmentation in mammals: in Biology of Normal and Abnormal Melanocytes. Ed. T. Kawamura, T.B. Fitzpatrick and M. Seiji, University Park Press, Baltimore. p.99.

QUEVEDO, W.C. Jr., SZABO, G., VIRKS, J., SINESI, S.J. (1965) Melanocyte populations in UV-irradiated human skin. Journal of Investigative Dermatology, 45, 295.

RAPER, H.S. (1928) The aerobic oxidases. Physiological Reviews, 8, 245.

RAPPAPORT, H. (1966) Tumours of the haematopoietic system: in Atlas of Tumour Pathology section 3 part 8. Armed Forces Institute of Pathology, Washington, D.C.

RAPPAPORT, H., NAKAI, T., SWIFT, H. (1963) The fine structure of normal and neoplastic melanocytes in the Syrian hamster, with particular reference to carcinogen-induced melanotic tumours. Journal of Cell Biology, 16, 171.

RAWLES, M.E. (1948) Origin of melanophores and their role in development of color patterns in vertebrates. Physiological Reviews, 28, 383.

REAMS, W.M., GRECO, P.P. (1969) An immunochemical study of the epidermal Langerhans cells of the mouse. Virginia Journal of Medical Science, 20, 113.

REAMS, W.M., GRECO, P.P. (1970) Cytoxan effects on epidermal Langerhans cells. Virginia Journal of Medical Science, 21, 147.

REAMS, W.M., TOMPKINS, S.P. (1973) A developmental study of murine epidermal Langerhans cells. Developmental Biology, 31, 114.

REVEL, J.P., KARNOVSKY, M.J. (1967) Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. Journal of Cell Biology, 33, C7.

REYNOLDS, E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology, 17, 208.

RICHARDSON, K.C., JARRETT, L., FINKE, E.H. (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technology, 35, 313.

RICHTER, R. (1956) Studien zur neurohistologie der nervösen vegetativen peripherie der haut bei verschiedenen chronisch-infektösen granulomen, mit besonderer berücksichtigung der Langerhansschen zellen. Archiv für Klinische und Experimentelle Dermatologie, 202, 466

RIEHL, G. (1884) Zur kenntnis des pigmentes in menschlichen haar. Archiv für Dermatologie und Syphilis, 11,33.

RILEY, P.A. (1966) Esterase in epidermal dendritic cells of the mouse. British Journal of Dermatology, 78, 388.

RILEY, P.A. (1975) The Langerhans cell: in The Physiology and Pathophysiology of the Skin, Vol. 3. Ed. A. Jarrett, Academic Press, London. p.1199.

ROSTON, S. (1960) Reaction of the sulfhydryl group with an oxidation product of B-3, 4-dihydroxyphenyl-L-alanine. Journal of Biological Chemistry, 235, 1002.

RUPEC, M. (1973) Die ultrastruktur der epidermis: in Haut und Anhangsgebilde. Spezielle Histopathologie Schnyder, U.W. vol.Ed. Springer Verlag, Berlin. p. 752.

SAGEBIEL, R.W. (1972) In vivo and in vitro uptake of ferritin by Langerhans cells of the epidermis. Journal of Investigative Dermatology, 58, 47.

SAGEBIEL, R.W.M., CLARKE, M.A., HUTCHENS, L.H. (1971) Dendritic cells in oral epithelium: in Current Concepts of the Histology of Oral Mucosa. Ed. C. Squier and J. Meyer, Charles C. Thomas, Springfield, Illinois.

SAGEBIEL, R.W., REED, T.H. (1968) Serial reconstruction of the characteristic granule of the Langerhans cell. Journal of Cell Biology, 36, 595.

SANEL, F.T., SERPICK, A.A. (1970) Plasmalemmal and subsurface complexes in human leukaemic cells: membrane bonding by zipper like junctions. Science, 168, 1458.

SCHELLANDER, F., WOLFF, K. (1967) Zur autoradiographischen markierung von Langerhans zellen mit 3H thymidin. Archiv fur Klinische und Experimentelle Dermatologie, 230, 140.

SCHENK, (1975) Melanocyten, Langerhanssche und merkelsche zellen in oralen epithelien. Acta Oto-Laryngologica, 80, 301.

SCHROEDER, H.E., THEILADE, J. (1966) Electron microscopy of normal human gingival epithelium. Journal of Periodontal Research, 1, 95.

SEIJI, M., FITZPATRICK, T.B., BIRBECK, M.S.C. (1961) The melanosome; a distinctive subcellular particle of mammalian melanocytes and the site of melanogenesis. Journal of Investigative Dermatology, 36, 243.

SHAMOTO, M., KAPLAN, C., KATOH, A. (1971) Langerhans cell granules in human hyperplastic lymph nodes. Archives of Pathology, 92, 46.

SHEA, M.S. (1971) Lanthanum staining of the surface coat of cells. Its enhancement by the use of fixatives containing alcian blue or cetylpyridinium chloride. Journal of Cell Biology, 51, 611.

SIEKEVITZ, P., PALADE, G.E. (1958) A cytochemical study of the pancreas of the guinea pig II. Functional variations in the enzymatic activity of microsomes. Journal of Biophysical and Biochemical Cytology, 4, 309.

SILBERBERG, I. (1971) Studies by electron microscopy of epidermis after topical application of histochemical findings in epidermal cells of human subjects who do not show reactions to mercuric chloride (0.1%). Journal of Investigative Dermatology, 56, 47.

SILBERBERG, I. (1973) Apposition of mononuclear cells to Langerhans cells in contact allergic reactions. Acta Dermato-Venereologica, 53, 1.

SILBERBERG, I., BAER, R.L., ROSENTHAL, S.A. (1974) The role of Langerhans cells in contact allergy. Acta Dermato-Venereologica, 54, 321.

SILBERBERG, I., BAER, R.L., ROSENTHAL, S.A., THORBECKE, G.J., BEREZOWSKY, V. (1975) Dermal and intravascular Langerhans cells at sites of passively induced allergic contact sensitivity. Cellular Immunology, 18, 435.

SILVERS, W.K. (1957) A histological and experimental approach to determine the relationship between gold-impregnated dendritic cells and melanocytes. American Journal of Anatomy, 100, 225.

SMITH, R.E., FARQUHAR, M.G. (1966) Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. Journal of Cell Biology, 31/2, 319.

SNELL, R.S. (1965) An electron microscopic study of the dendritic cells in the basal layer of guinea pig epidermis. Zeitschrift für Zellforschung und Mikroskopische Anatomie, 66, 457.

STUART, A.E. (1975) Malignant Lymphoma. Journal of the Royal College of Surgeons of Edinburgh, 20, 332.

SWANSON, J.L., WAYTE, D.M., HELWIG, E.B. (1968) Ultrastructure of halo nevi. Journal of Investigative Dermatology, 50, 434.

SZABO, G. (1967a) The regional anatomy of the human integument with special reference to the distribution of hair follicles, sweat glands and melanocytes. Philosophical Transactions of the Royal Society, Series B, 252, 447.

SZABO, G.S. (1967b) Photobiology of melanogenesis: Cytological aspects with special reference to differences in racial coloration: in Advances in Biology of Skin, Vol. 8, The Pigmentary System. Ed. W. Montagna and F. Hu, Pergamon Press, Oxford. p.379.

SZABO, G., GERALD, A.B., PATHAK, M.A., FITZPATRICK, T.B. (1969) Racial differences in the fate of melanosomes in human epidermis. Nature, 222, 1081.

SZEKERES, L. (1970) A.T.P.'ase and Langerhans cells investigated in psoriasis. Zeitschrift fur Haut und Geschlechts-Krankleiten, 45, 611.

TARNOWSKI, W.M., HASHIMOTO, K. (1967) Langerhans cell granules in histiocytoses X. The epidermal Langerhans cell as a macrophage. Archives of Dermatology, 96, 298.

TODA, K., FITZPATRICK, T.B. (1971) The origin of melanosomes: in Biology of Normal and Abnormal Melanocytes. Ed. T. Kawamura and T.B. Fitzpatrick and M. Seiji, University Park Press, Baltimore. p. 265.

TODA, K., PATHAK, M.A., PARRISH, J.A., FITZPATRICK, T.B., QUEVEDO, W.C. Jr. (1972) Alteration of racial differences in melanosome distribution in human epidermis after exposure to ultraviolet light. Nature New Biology, 236, 143.

TURIAF, M.J., BASSET, F. (1966) Deux nouveaux cas d'histiocytose X à localisations pulmonaires et osseuses avec présence dans les lésions granulomateuses de particules tubulaires intracytoplasmiques suggérant une structure virale. Société Médicale de Hôpitaux de Paris, 117, 373.

TUSQUES, J., PRADAL, G. (1969) Analyse tridimensionnelle des inclusions recontrées dans les histiocytes de l'histiocytose X en microscopie électronique comparaison avec les inclusions des cellules de Langerhans. Journal de Microscopie, 8, 113.

WEAKLEY, B.S. (1972) Rinsing and holding buffers: in A Beginners Handbook in Biological Electron Microscopy. Ed. B.S. Weakley, Churchill Livingstone, Edinburgh. p.200.

WELLINGS, S.R., SIEGEL, B.V. (1963) Electron microscopic studies of the subcellular origins and ultrastructure of melanin granules in mammalian melanosomes. Annals of the New York Academy of Sciences, 100, 548.

WIEDMANN, A. (1952) Zur frage der sogenannten Langerhans zellen der haut. Hautarzt, 3, 249.

WINKELMANN, R.K. (1960) Nerve endings in normal and pathologic skin. Contributions to the Anatomy of Sensation. Charles C. Thomas Publisher, Springfield, Illinois. p.30.

WOLFF, K. (1967) The fine structure of the Langerhans cell granule. Journal of Cell Biology, 35, 468.

WOLFF, K. (1972) The Langerhans cell: in Current Problems in Dermatology, Vol. 4. Ed. J.W.H. Mali, S. Karger, Basel. p.79.

WOLFF, K., HÖNIGSMANN, H. (1971) Permeability of the epidermis and phagocytic activity of keratinocytes. Journal of Ultrastructural Research, 36, 176.

WOLFF, K., HÖNIGSMANN, H. (1972) Are melanosome complexes lysosomes? Journal of Investigative Dermatology, 59, 170.

WOLFF, K., HÖNIGSMANN, H. (1973) Personal communication.

WOLFF, K., JIMBOW, K., FITZPATRICK, T.B. (1972) The formation of melanosome complexes: a size dependent phenomenon. Journal of Investigative Dermatology, 58, 257.

WOLFF, K., KONRAD, K. (1972) Phagocytosis of latex beads by epidermal keratinocytes in vivo. Journal of Ultrastructural Research, 39, 262.

WOLFF, K., SCHREINER, E. (1968) An electron microscopic study of the extraneous coat of keratinocytes and the intercellular space of the epidermis. Journal of Investigative Dermatology, 51, 418.

WOLFF, K., SCHREINER, E. (1970a) Epidermal lysosomes. Electron microscopic-cytochemical studies. Archives of Dermatology, 101, 276.

WOLFF, K., SCHREINER, E. (1970b) Uptake, intracellular transport and degradation of exogenous protein by Langerhans cells. An electron microscopic-cytochemical study using peroxidase as tracer substance. Journal of Investigative Dermatology, 54, 37.

WOLFF, K., SOLLEREDER, B. (1969) Intranuclear and extracellular Langerhans cell granules. Journal of Investigative Dermatology, 52, 424.

WOLFF, K., WINKELMANN, R.K. (1967a) Quantitative studies on the Langerhans cell population of guinea pig epidermis. Journal of Investigative Dermatology, 48, 504.

WOLFF, K., WINKELMANN, R.K. (1967b) Nonpigmentary enzymes of the melanocyte-Langerhans cell system: in Advances in Biology of the Skin Vol. VIII, The Pigmentary System. Ed. W. Montagna and F. Hu, Pergamon Press, Oxford. p. 135.

WONG, Y.C., BUCK, R.C. (1971) Langerhans cells in epidermoid metaplasia. Journal of Investigative Dermatology, 56, 10.

ZELICKSON, A.S. (1965) The Langerhans cell. Journal of Investigative Dermatology, 44, 201.

ZELICKSON, A.S. (1967a) Melanocyte, melanin granule and Langerhans cell: in Ultrastructure of Normal and Abnormal skin. Ed. A.S. Zelickson, Lea and Febiger, Philadelphia. p.163.

ZELICKSON, A.S. (1967b) The pigmented basal cell carcinoma. Archives of Dermatology, 96, 524.

ZELICKSON, A.S., HARTMANN, J.F. (1961) The fine structure of the melanocyte and melanin granule. Journal of Investigative Dermatology, 36, 23.

ZELICKSON, A.S., MOTTAZ, J.H. (1968) Epidermal dendritic cells. A quantitative survey. Archives of Dermatology, 98, 652.

ZELICKSON, A.S., MOTTAZ, J.H. (1970) The effect of sunlight on human epidermis. A quantitative electron microscopic study of dendritic cells. Archives of Dermatology, 101, 312.

ZELICKSON, A.S., MOTTAZ, J.H., HUNTER, J.A.A. (1972) An electron microscopic study on the effect of ultraviolet irradiation on human skin: 1. Autophagy and melanosome degradation in melanocytes: in Pigmentation. Its Genesis and Biologic Control. Ed. V. Riley, Appleton-Century Crofts, New York. p.445.

ACKNOWLEDGEMENTS

It is a pleasure to thank

D.J. FAIRLEY, Department of Dermatology, University of Edinburgh, for his skilful technical assistance. It would be difficult to over-emphasize the value of his help in this thesis.

DR. MARY K. MACDONALD, Department of Pathology, University of Edinburgh, for her enthusiastic help as University Adviser for this thesis. Her constructive criticisms have been invaluable.

MRS. JENNY STEWART, good friend of our family, for her outstanding ability to transcribe my indecipherable hand writing into neat type.

PROFESSOR P.J. HARE, Department of Dermatology, University of Edinburgh, for his continued encouragement, and for provision of facilities to carry out this work. I am also indebted to him for translating numerous German papers included in this thesis.

PATIENTS and STAFF, for allowing me to photograph lesions and to take biopsies of normal skin.

MRS. EVA MCVITTIE, Department of Dermatology, University of Edinburgh, for her everyday encouragement and for her collaboration in the work reported in Chapters V, VI, and VIII. She has also helped to bring some order to my filing system of references.

DR. A.S. ZELICKSON, Department of Dermatology, University of Minnesota, U.S.A., for introducing me to the world of fine structure in such a way that I have enjoyed applying my research interests in that direction ever since.

J.S. and MRS. LENORE MOTTAZ, Department of Dermatology, University of Minnesota, U.S.A., for the hours that they both spent introducing me to the basic techniques of electron microscopy. Their good advice was, in turn, forwarded to David Fairley, and much of the routine methodology used in our unit stems from Minneapolis.

PROFESSOR K. WOLFF, Department of Experimental Dermatology, University of Vienna, Austria, for allowing me to visit his unit. The very high standard set in that unit and his immense enthusiasm recharged my own batteries. Both our techniques, and the application of them, have improved since my visit to Vienna.

DR. G.W. BEVERIDGE, DR. J.A. SAVIN and Colleagues in the Department of Dermatology, University of Edinburgh, for their continued interest in my work, and for allowing me to study patients under their care.

MR. A.C. BUCHAN and STAFF, Department of Plastic Surgery, University of Edinburgh, for allowing me to study patients with malignant melanoma under their care.

PROFESSOR SIR MICHAEL WOODRUFF, MR. A.J. DUFF and STAFF, Department of Surgery, University of Edinburgh, for their co-operation, and for allowing me to study patients under their care.

PROFESSOR A.P.M. FORREST and STAFF, Department of Clinical Surgery, University of Edinburgh, for their co-operation, and for allowing me to study patients under their care.

SURGEONS of EDINBURGH AND KIRKCALDY, for allowing me to study patients with malignant melanoma under their care.

PROFESSOR A.R. CURRIE, Department of Pathology, University of Edinburgh and PROFESSOR G.J. ROMANES, Department of Anatomy, University of Edinburgh for allowing me to use the electron microscopes in their departments.

R. DONALDSON, Department of Pathology, University of Edinburgh and R. MACDOUGALL, Department of Anatomy, University of Edinburgh for all their help, and for coming to the rescue when technical problems arose in the use of their electron microscopes.

DR. D.M. DOUGLAS, Department of Child Life and Health, University of Edinburgh for allowing me to study the patient reported in Chapter VII.

DR. J.D. MCGREGOR, DR. G. SCLARE, DR. A. SHIVAS and STAFF, Department of Pathology, University of Edinburgh, for their continued interest and co-operation in providing pathological material studied in this thesis. Dr. Shivas also collaborated in the study of the patient reported in Chapter IX.

DR. J.S. COMAISH, Department of Dermatology, University of Newcastle, for collaboration in the work described in Chapters V and VI.

DR. S. BLEEHAN, Rupert Hallam Department of Dermatology, University of Sheffield; DR. A.J. COCHRAN, University Department of Pathology, Glasgow; DR. R. MACKIE, University Department of Dermatology, Glasgow and DR. W.D. PATTERSON

and DR. S. ZAYNOUN, Department of Dermatology, University of Edinburgh for their collaboration in the study described in Chapter VIII.

PROFESSOR JOSEF PIEPER, University of Munster, Munich, Germany for his help which is described in Chapter I.

THE STAFF OF THE DEPARTMENT OF MEDICAL ILLUSTRATION, University of Edinburgh, and in particular J. PAUL and I. LENNOX for their help in producing figures, tables and light microscopic photographs included in this thesis.

THE STAFF OF THE CENTRAL MEDICAL LIBRARY, University of Edinburgh, for their help in searching for and obtaining difficult references.

THE SCOTTISH HOSPITAL ENDOWMENT RESEARCH TRUST for providing the finances which enabled the studies reported in Chapters IV, V, VI and VII to be carried out.

THE MEDICAL RESEARCH COUNCIL for providing a grant which enabled the work reported in Chapters VIII and IX to be carried out.

RUTH for her continued encouragement and help, and

RUTH AND THE CHILDREN. They have put up with an absent husband and father with much understanding.

THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 54, No. 3

Printed in U.S.A.

MELANOGENESIS: ULTRASTRUCTURAL HISTOCHEMICAL OBSERVATIONS ON ULTRAVIOLET IRRADIATED HUMAN MELANOCYTES*

JOHN A. A. HUNTER, M.B., CH.B., M.R.C.P.E.,† JESS H. MOTTAZ, B.S. AND ALVIN S. ZELICKSON, M.D.

ABSTRACT

Ultrastructural DOPA and tyrosine reactions have been used to provide information regarding subcellular sites of melanogenesis within the melanocyte following ultraviolet irradiation of normal human skin. Our observations indicate that the earliest premelanosomes arise from all parts of the Golgi apparatus as well as from a Golgi associated system of smooth endoplasmic reticulum (GERL). The time interval following irradiation influences the site of enzyme activity. The role of the Golgi apparatus in melanogenesis is discussed and special attention is drawn to the fact that some premelanosomes frequently show no tyrosinase activity, even though such enzyme activity may be present in neighboring vesicles.

Melanin is synthesized in mammalian skin in special secretory cells known as melanocytes. These cells contain the enzyme tyrosinase which is responsible for the conversion of tyrosine to dihydroxyphenylalanine (DOPA) and dopaquinone (1), and the conversion of 5-6 dihydroxyindole to indole-5,6-quinone (2). This latter monomer is then oxidized and polymerized to form melanin which is attached to the protein matrix of the melanosome by various linkages.

Much knowledge about the subcellular localization of melanin biosynthesis has been gained using recently available techniques such as ultracentrifugal separation of cell particles, density gradient centrifugation and electron microscopic monitoring of the separated cell fractions (3, 4, 5, 6, 7). It is generally accepted that tyrosinase is synthesized on the ribosomes, transferred via the rough endoplasmic reticulum to the Golgi apparatus where it is assembled into units each of which is surrounded by a smooth surfaced membrane to form a vesicle. The melanin polymer is then gradually deposited within this vesicle on an inner membranous structure. Various stages (premelanosomes) between the smallest vesicle containing tyrosinase and the fully developed melanosome have been described (8). The ultrastructural morphology of human melanomas is compatible with this concept (9) and, in mouse melanomas, such a pathway has been supported by both *in vivo* and *in vitro* ultrastructural autoradiographic studies (10, 11, 12). Contemporary views on the subcellular localization of melanin biosynthesis have been summarized in two recent reviews (13, 14).

Thus, our present knowledge on the subcellular localization of melanin biosynthesis has been largely guided by work on the abnormal neoplastic pigment producing cells of mice, using the above mentioned procedures. Such methods are not without obvious intrinsic limitations which include damage to isolated cell organelles and a degree of interfractional contamination.

This study was undertaken to investigate the subcellular sites of melanogenesis in human melanocytes using the ultrastructural DOPA reaction described by Mishima (15), and the light microscopic tyrosine reaction of Fitzpatrick *et al.* (16) adapted for ultrastructural usage. Particular attention has been paid to the region of the Golgi apparatus, which is shown schematically in Figure 7. The term saccule is preferred to cisterna, and the two faces of the apparatus have been called vesicular and vacuolar rather than forming and ma-

* From the Department of Dermatology, University of Minnesota Medical School, Minneapolis, Minnesota 55455. (Reprint requests to Dr. Zelickson.)
† Present address: Department of Dermatology, Royal Infirmary, Edinburgh, Scotland.
Supported by Research Training Grant #AM-05560-03 USPHS.
Presented at the Spring meeting of the Society for Investigative Dermatology, Inc., May 3, 1969, Atlantic City, New Jersey.
Received May 5, 1969; accepted for publication September 30, 1969.

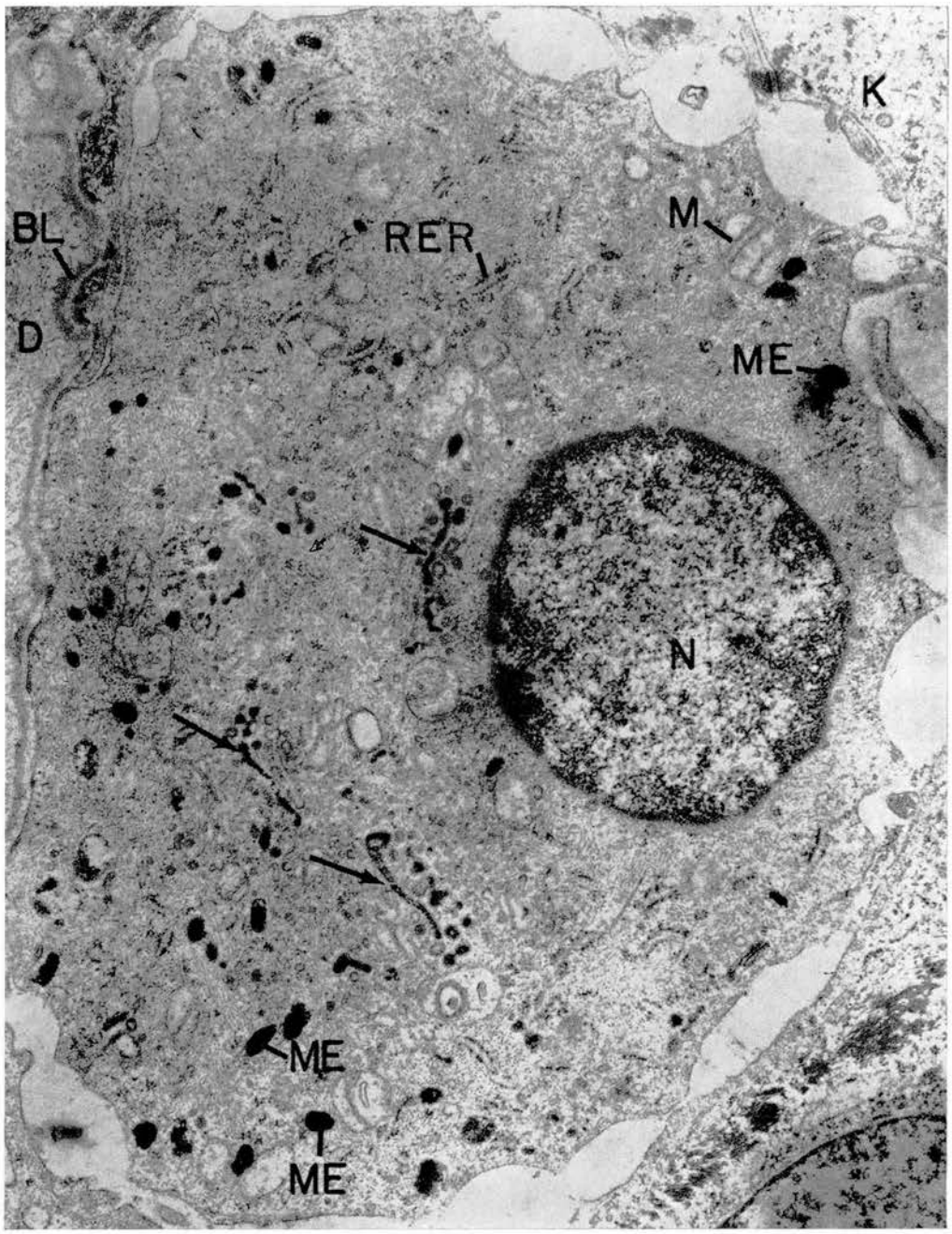


FIG. 1. 72 hrs following ultraviolet irradiation. Incubation: tyrosine. Melanocyte on the basal lamina (BL). Linear reaction product (arrows) together with vesicles containing reaction product are seen in close association with the Golgi apparatus. Numerous vesicles, of varying sizes and containing reaction product, are seen between those associated with the Golgi complex and the melanosomes (ME). K. Keratinocyte. M. Mitochondrion. RER. Rough endoplasmic reticulum. N. Nucleus. D. Dermis. × 18,200.

ture (see discussion). As the Golgi apparatus of the melanocyte frequently shows no convex or concave face these adjectives are not used. The system of smooth endoplasmic reticulum closely associated with the Golgi apparatus is referred to as GERL according to the terminology of Novikoff (17, 18, 19).

MATERIALS AND METHODS

The untanned skin of the upper forearm of a 30 year old Caucasian male was selected as the test site. The minimal erythema dose (MED) for this area using a hot quartz lamp source (Burdick Company, Milton, Wisconsin. Type QA 450. Peak transmission at the 309nm and 313nm bands) was established. An area of approximately 2 cm^2 was then irradiated with a dose equivalent to 6 X MED. This caused visible erythema in 1–2 hours following irradiation and slight clinical edema by 24 hours, when the reaction appeared to be at its height. Vesiculation was never noted and the treated area always showed residual pigmentation when the erythema had disappeared (approx. 120 hrs. following irradiation). Tissue from the test area was taken with a 3mm punch biopsy after irradiation and the following intervals after: 2 hr, 6 hr, 24 hr, 72 hr and 120 hr. Ultrastructural DOPA, tyrosine and control reactions were carried out on each biopsy specimen.

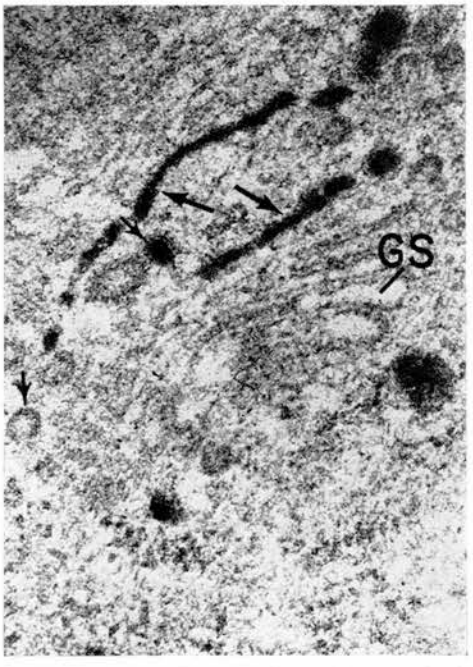


FIG. 2. 72 hrs following ultraviolet irradiation. Incubation: tyrosine. Golgi region of a melanocyte. Large arrows point to linear reaction product in Golgi associated endoplasmic reticulum (GERL) (upper left), and in the peripheral saccule at the vesicular face of the Golgi apparatus (lower right). They are separated by vesicles containing reaction product (small arrows). GS. Golgi saccules. × 59,300.

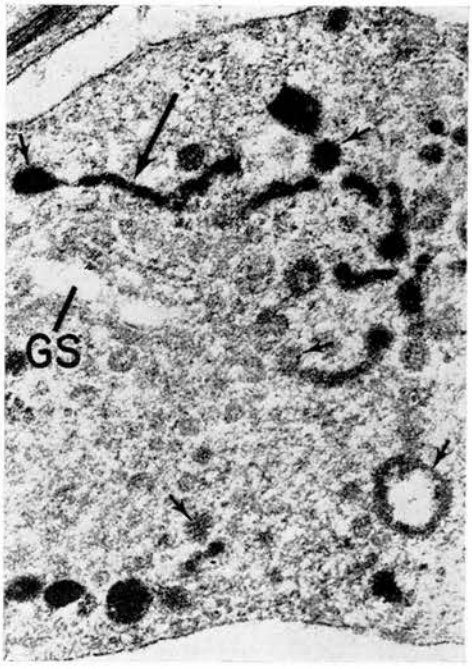


FIG. 3. 72 hrs following ultraviolet irradiation. Incubation: tyrosine. Golgi region of a melanocyte. Large arrow points to reaction product in Golgi associated endoplasmic reticulum (GERL). Note numerous vesicles containing reaction product (small arrows), some of which appear to be budding from the linear reaction product. GS. Golgi saccule. × 46,100.

Ultrastructural DOPA reaction. The tissue was fixed in cold 2.5% glutaraldehyde 0.1M phosphate buffer, pH 6.8 and sliced into thin sections of approximately 100μ thick after 90 mins. Total fixation time was 2–3 hrs. The sections were then thoroughly rinsed in 0.1M phosphate buffer, pH 6.8, and transferred to the incubating medium containing 0.1% L-3, 4-DOPA freshly prepared in 0.1M phosphate buffer, pH 6.8. The slices were kept in this overnight at 3–4° C and then transferred to fresh incubating medium at 37° C. After 7 hours incubation the sections were rinsed and post-fixed in cold buffered 1% osmium tetroxide for 1 hr.

Ultrastructural tyrosine reaction. The tissue was again fixed in cold 2.5% glutaraldehyde-0.1M phosphate buffer, pH 6.8, and sliced into thin sections of approximately 100μ after 90 mins. Total fixation time was 2–3 hrs. Following rinsing in 0.1M phosphate buffer, pH 6.8, the sections were transferred to the incubation medium containing 0.05% L-tyrosine freshly prepared in 0.1M phosphate buffer, pH 6.8. After incubation overnight at 3–4° C the

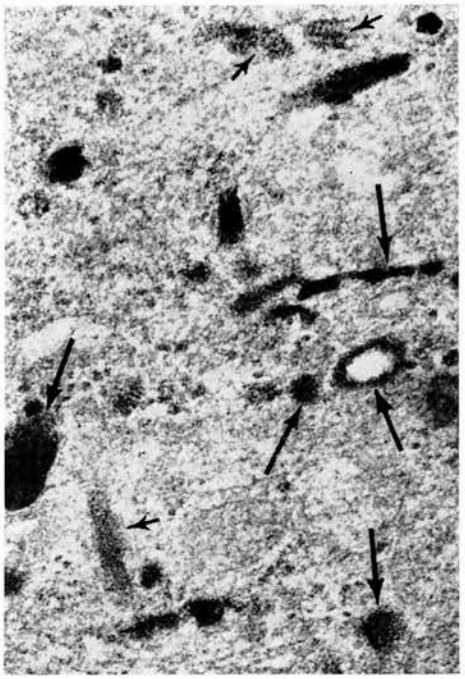


FIG. 4. 72 hrs following ultraviolet irradiation. Incubation: DOPA. Note that, in spite of linear and vesicular reaction product (long arrows), there are areas showing the typical matrix pattern of premelanosomes containing no reaction product (short arrows). × 50,000.

sections were transferred to fresh incubation medium and incubated at 37° C for 24 hrs. The sections were then rinsed and postfixed in cold osmium tetroxide.

Control reaction. This was carried out exactly as for the DOPA and tyrosine reactions except that the incubation medium did not contain any substrate in the 0.1M phosphate buffer, pH 6.8.

Following postfixation with osmium tetroxide all sections were dehydrated in alcohol and embedded in epon according to standard procedures. Thin sections, cut with an LKB Ultratome, were stained with uranyl acetate followed by lead citrate and viewed with a RCA EMU-3 electron microscope.

RESULTS

Following ultraviolet irradiation the DOPA and tyrosine reaction product is specifically deposited within the melanocytes of the epidermis (Figs. 1–6). Keratinocytes and Langerhans cells do not contain reaction product. Irradiated melanocytes incubated in phosphate buffer do not contain reaction product, while non irradiated melanocytes, incubated in DOPA or tyrosine, may contain vesicular but not linear reaction product (see below).

The cellular and subcellular localization and nature of the reaction product using both substances as substrates are essentially identical. With the methods used, reaction product is more commonly seen when DOPA is the substrate, though subcellular morphology has not been so well preserved as when tyrosine is used. For this reason, the micrographs (except Figs. 4 and 6) are from sections incubated in tyrosine.

Linear reaction product has been seen in: 1) Smooth endoplasmic reticulum not clearly associated with the Golgi apparatus; 2) Smooth endoplasmic reticulum closely associated with the Golgi apparatus (GERL), this being the most common site (Figs. 1, 2, 3, 5); and 3) Golgi saccules (Figs. 2, 6). The linear reaction product often appears to have broken down into small vesicles containing reaction product (Figs. 2, 3, 5, 6), and frequently similar vesicles bud from the ends of sides of

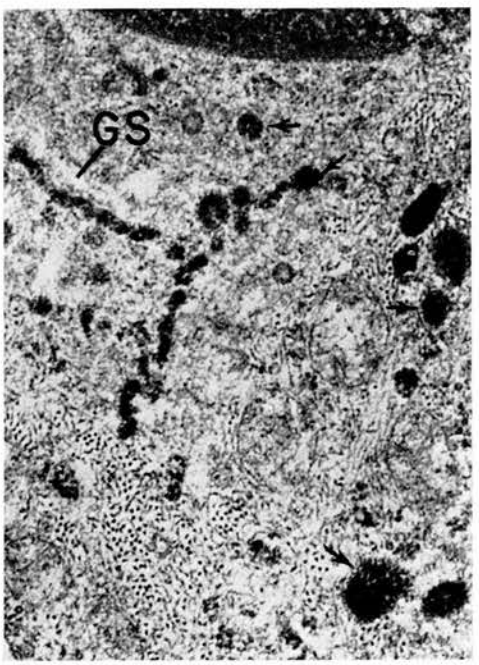


FIG. 5. 72 hrs following ultraviolet irradiation. Incubation: tyrosine. Golgi region of a melanocyte. The linear reaction product appears as numerous small vesicles. The small arrows point to vesicles, of varying sizes, containing reaction product. GS. Golgi saccule. × 42,200.

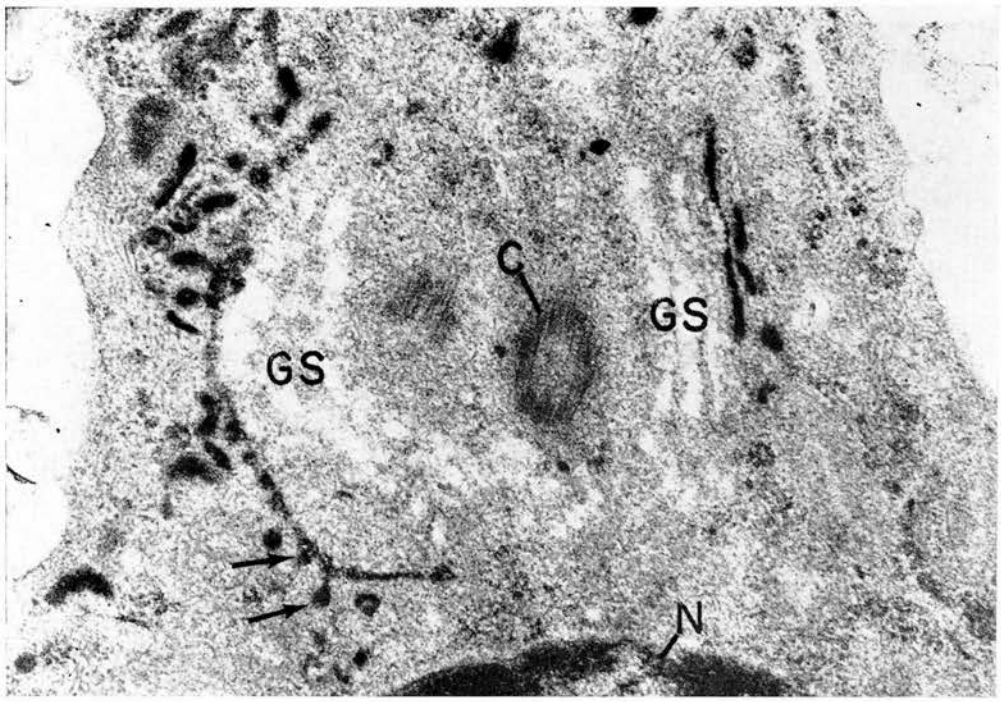


FIG. 6. 72 hrs following ultraviolet irradiation. Incubation: DOPA. Golgi region of a melanocyte. Reaction product is seen in the peripheral saccules at the vesicular face of the Golgi complex, with stacking on the right. Note small vesicles containing reaction product budding from some places (small arrows). N. Nucleus. C. Centriole. GS. Golgi saccule. × 46,000.

saccules containing reaction product (Figs. 1–6).

Vesicular reaction product has been seen in: 1) Golgi vesicles and vesicles closely associated with Golgi apparatus (GERL) (Figs. 1–6). It is often impossible to differentiate between these. 2) Intracytoplasmic vesicles of all sizes ranging from 50 mμ in diameter to 500 mμ in diameter (Figs. 1–6).

At times, melanocytes demonstrating reaction product contain premelanosomes in which there is no deposition of the reaction product (Fig. 4).

Rarely, a diffuse darkening of the cytoplasm of DOPA incubated melanocytes was noted. This seemed to be attributable to section thickness, but also tended to occur at times when numerous vesicles containing reaction product were present (e.g., 120 hrs after irradiation).

The subcellular localization of the reaction product (summarized in Table I) is dependent on the interval following irradiation. Linear reaction product was not seen until 24 hrs following irradiation. At this time a higher proportion of the reaction product was present in a linear form in the smooth endoplasmic reticulum associated with the Golgi apparatus, while at 120 hrs post irradiation more is seen in discrete vesicles of all sizes.

Reaction product was not noted in mast cells or melanophages. Grids with numerous melanocytes containing reaction product often had mast cells with characteristic "finger printing" of their granules (indicating no reaction product within) and no reaction product in other areas.

DISCUSSION

Reaction product, of similar nature and localization, has been observed in ultraviolet irradiated melanocytes when DOPA and tyrosine are used as substrates. Lerner *et al.* (20) emphasized that at a pH above 7.0 DOPA is oxidized in the presence of oxygen without the need for a catalyst, and therefore suggested that an incubation medium with a pH of 6.8 would minimize such auto-oxidation. It

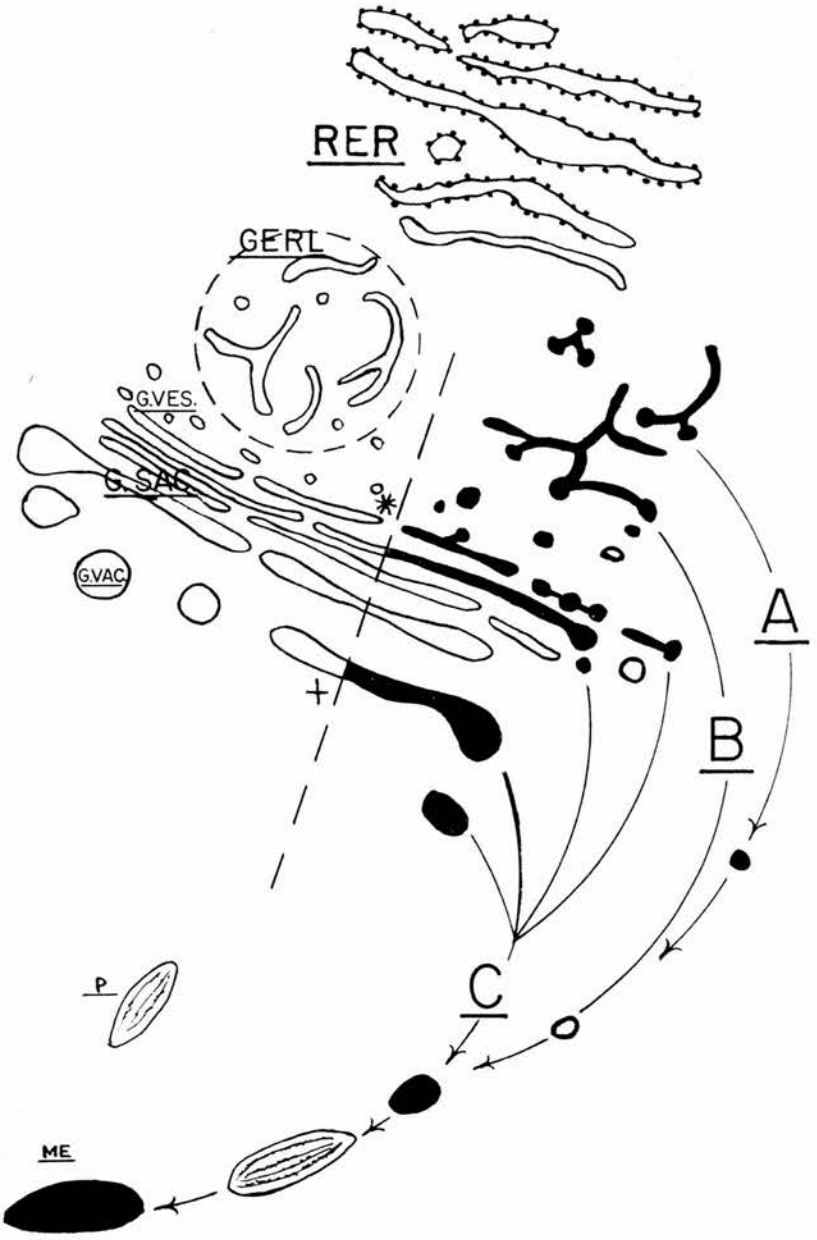


FIG. 7. Composite diagram of the Golgi region in a melanocyte. To the left of the interrupted line the Golgi apparatus has been labelled according to the terminology used in this text: G. VES. Golgi vesicles. G. SAC. Golgi saccules. G. VAC. Golgi vacuoles. * Vesicular face of the apparatus. + Vacuolar face of the apparatus. GERL Golgi associated system of smooth endoplasmic reticulum. RER. Rough endoplasmic reticulum. The subcellular localization of reaction product is illustrated at the right of the interrupted line. The arrows indicate suggested pathways in the development of the melanosome (ME). A. From smooth endoplasmic reticulum. B. From smooth endoplasmic reticulum associated with the Golgi Complex (GERL). C. From all parts of the Golgi apparatus. Of these, B pathway appears to be the most common. Note the structures resembling the matrix of premelanosomes (P) which contain no reaction product, even though it is present in surrounding vesicles.

TABLE I

Cellular and subcellular localization of reaction product

Location	Incubated in DOPA or tyrosine						Incubated in buffer		
	Non-irrad.	2 hr.	6 hr.	24 hr.	72 hr.	120 hr.	Non-irrad.	24 hr.	72 hr.
Epidermis									
Melanocyte									
Cytoplasm (darkening)	−	−	−	−	−	+	−	−	−
Mitochondrion	−	−	−	−	−	−	−	−	−
Smooth endoplasmic reticulum	−	−	−	+	+	−	−	−	−
Golgi region									
a) Associated smooth endoplasmic reticulum (GERL)	−	−	−	++	+++	+	−	−	−
b) Peripheral saccule and vesicles at the vesicular face	−	−	−	+	+++	++	−	−	−
c) Saccules	−	−	−	+	+	−	−	−	−
d) Peripheral saccule and vacuoles at the vacuolar face	−	−	−	−	+	+	−	−	−
Intracytoplasmic vesicles (100–500 mμ)	+	+	+	+	+++	+++	−	−	−
Langerhans cell	−	−	−	−	−	−	−	−	−
Keratinocyte	−	−	−	−	−	−	−	−	−
Dermis									
Mast cell	−	−	−	−	−	−	−	−	−
Melanophage	−	None seen	−	−	−	None seen	−	−	−

− No reaction product noted, + Reaction product rarely noted, ++ Reaction product commonly noted, +++ Reaction product often noted.

is, however, generally considered (21) that the reaction product derived from the tyrosine in an *in vitro* procedure, such as we have used, is more specific for the demonstration of tyrosinase. As there was no significant difference either in localization or nature of the reaction product derived from both substrates, it seems probable that they both represent sites of tyrosinase activity.

Thus it has been shown that tyrosinase activity can be satisfactorily demonstrated in discrete subcellular localizations within normal human melanocytes following ultraviolet irradiation. The ultrastructural histochemical technics used have allowed a reasonable compromise between the demonstration of enzyme activity and adequate tissue morphology. As a result a diagram illustrating the subcellular localization of reaction product has been composed (Fig. 7). From this it can be seen that the Golgi region attains a central significance in the localization of reaction product.

Biochemical analysis, autoradiography and ultrastructural morphology have indicated that secretory proteins are concentrated and packaged at various sites within the Golgi region, depending on the cell type examined. Thus, this occurs in condensing vacuoles in guinea pig pancreas (22, 23), in vesicles derived from opposite faces of the Golgi apparatus in rabbit polymorphonuclear leukocytes (24), in vacuoles derived from Golgi saccules in rat anterior pituitary cells (25) and in a Golgi associated system of smooth endoplasmic reticulum in cells of rat adrenal medulla and mouse melanoma (26, 19). Our observations support the concept that tyrosinase may be packed in all of these regions in the human melanocyte to a lesser or greater degree.

At 24 and 72 hrs following ultraviolet ir-

radiation the reaction product is seen most often in the smooth endoplasmic reticulum related to the Golgi apparatus. Novikoff (17, 18, 19), on the basis of a high acid phosphatase content and its relationship with coated vesicles and lysosomes, considers this Golgi associated system of smooth endoplasmic reticulum to have a special functional significance. He has named it GERL to indicate that it is part of the endoplasmic reticulum (ER), closely related to the Golgi apparatus (G) and producing lysosomes (L). Serial sectioning has revealed GERL to be continuous with the rough endoplasmic reticulum (17) but has not yet established such a relationship with the Golgi apparatus, though this seems likely. Whatever the region may be called we can confirm its prime importance in the process of melanogenesis. Our observations suggest that most vesicles containing tyrosinase make their earliest appearance from this area, and vesicles of gradually increasing size are seen between here and the cell periphery. The production of such vesicles would not appear, in this instance, to involve the Golgi apparatus at all. It is difficult to define the mechanism whereby developing premelanosomes increase in size as vesicles containing reaction product have not been noted to fuse together.

Tyrosinase activity has also been detected within Golgi saccules, more often at the vesicular face (Figs. 2 and 6) than at the vacuolar face. Vesicles containing tyrosinase appear to bud from these regions as well (Fig. 6), indicating other pathways of premelanosomal production.

Knowledge about the function of the ubiquitous Golgi vesicles is now being broadened. Jamieson and Palade (27), in their studies on pancreatic cell secretion, showed that some are involved in protein transport from the rough endoplasmic reticulum to the Golgi saccules. Our work, like that of others (24, 19) suggests that some contain protein secretory products which have been packaged and are leaving the area. The terminology of the Golgi faces based on morphological appearance (vesicular and vacuolar) would therefore seem to be more appropriate than that implying functional characteristics (forming and mature) as originally suggested by Mollenhauer and Whaley (28).

Linear reaction product could not be demonstrated in the melanocytes which were not exposed to ultraviolet irradiation. The tyrosinase levels in these areas are apparently too low for detection by our technics. Ultraviolet irradiation causes a burst of tyrosinase activity of limited duration and the newly synthesized enzyme can be detected as indicated in Table I.

We have demonstrated, as have others (6, 19) clear evidence of tyrosinase activity in areas of the melanocyte where, *in vivo*, pigment is not normally seen. Some (29) believe that the melanin polymer is simply not detectable in its earliest forms, but the presence of an *in vivo* tyrosinase inhibitor (30) which may be lost in conditions of *in vitro* preparation could also provide a possible explanation. It is of interest, however, that in certain neoplastic melanocytes pigment deposition has been observed within the endoplasmic reticulum and GERL (19, 31).

REFERENCES

1. Mason, H. S.: The chemistry of melanin. III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. J. Biol. Chem., *172:* 83, 1948.
2. Riley, P. A.: Histochemical demonstration of melanocytes by the use of 5,6-diacetoxyindole as substrate for tyrosine. Nature, *213:* 190, 1967.
3. Baker, R. V., Birbeck, M. S., Blaschko, H., Fitzpatrick, T. B. and Seiji, M.: Melanin granules and mitochondria. Nature, *187:* 392, 1960.
4. Seiji, M., Fitzpatrick, T. B. and Birbeck, M. S. C.: The melanosome: A distinctive subcellular particle of mammalian melanocytes and the site of melanogenesis. J. Invest. Derm., *36:* 243, 1961.
5. Seiji, M., Fitzpatrick, T. B., Simpson, R. T. and Birbeck, M. S. C.: Chemical composition and terminology of specialized organelles (melanosomes and melanin granules) in mammalian melanocytes. Nature, *197:* 1082, 1963.
6. Seiji, M. and Iwashita, S.: Intracellular localization of tyrosinase and site of melanin formation in melanocyte. J. Invest. Derm., *45:* 305, 1965.
7. Ishikawa, K., Fitzpatrick, T. B. and Szabo, G.: Some recent studies on the subcellular unit of melanin biosynthesis, the melanosome. *Structure and Control of the Melanocyte.* Eds., Della Porta, G. and Mühlbock, O., Springer-Verlag New York, Inc., New York, 1966.
8. Della Porta, G. and Mühlbock, O.: *Structure and Control of the Melanocyte.* Springer-Verlag New York, Inc., New York, 1966.
9. Wellings, S. R. and Siegel, B. V.: Electron microscopic studies of the subcellular and ultrastructure of melanin granules in mam-

malian melanomas. Ann. N. Y. Acad. Sci., *100:* 548, 1963.
10. Nakai, T. and Shubik, P.: Electron microscopic radioautography: The melanosome as a site of melanogenesis in neoplastic melanocytes. J. Invest. Derm., *43:* 267, 1964.
11. Blois, M. S. and Kallman, R. F.: The incorporation of C^{14} from 3,4-dihydroxyphenylalanine-2′-C^{14} into the melanin of mouse melanomas. Cancer Res., *24:* 863, 1964.
12. Zelickson, A. S. Hirsch, H. M. and Hartman, J. F.: Localization of melanin synthesis. J. Invest. Derm., *45:* 458, 1965.
13. Fitzpatrick, T. B., Miyamoto, M. and Ishikawa, K.: The evolution of concepts of melanin biology. Arch. Derm., *96:* 305, 1967.
14. Duchon, J., Fitzpatrick, T. B. and Seiji, M.: Melanin 1968: Some definitions and problems, pp. 6–33, *Year Book of Dermatology*. Eds., Kopf, A. W. and Andrade, R., Year Book Medical Publishers, Inc., Chicago. 1967–1968 Series.
15. Mishima, Y.: Electron microscopy of melanin synthesis in intradermal nevus cells. J. Invest. Derm., *39:* 369, 1962.
16. Fitzpatrick, T. B., Becker, S. W. Jr., Lerner, A. B. and Montgomery, H.: Tyrosinase in human skin: demonstration of its presence and of its role in human melanin formation. Science, *112:* 223, 1950.
17. Novikoff, A. B., Roheim, P. S. and Quintana, N.: Changes in rat liver cells induced by orotic acid feeding. Lab. Invest., *15:* 27, 1966.
18. Novikoff, A. B.: Enzyme localization and ultrastructure of neurons, and lysosomes in nerve cells, p. 255, *The Neuron*. Ed., Hyden, H., Elsevier Publishing Company, Amsterdam, 1967.
19. Novikoff, A. B., Albala, A. and Biempica, L.: Ultrastructural and cytochemical observations on B-16 and Harding-Passey mouse melanomas. The origin of premelanosomes and compound melanosomes. J. Histochem. Cytochem., *16:* 299, 1968.
20. Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Soumerson, W. H.: Mammalian tyrosinase: preparation and properties. J. Biol. Chem., *178:* 185, 1949.
21. Fitzpatrick, T. B. and Kukita, A.: Tyrosinase activity in vertebrate melanocytes, p. 489, *Pigment Cell Biology*. Ed., Gordon, M., Academic Press Inc., New York, 1959.
22. Caro, L. G. and Palade, G. E.: Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study. J. Cell Biol., *20:* 473, 1964.
23. Jamieson, J. D. and Palade, G. E.: Intracellular transport of secretory proteins in the pancreatic exocrine cell. II Transport to condensing vacuoles and zymogen granules. J. Cell Biol., *34:* 597, 1967.
24. Bainton, D. F. and Farguhar, M. G.: Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. J. Cell Biol., *28:* 277, 1966.
25. Smith, R. E. and Farguhar, M. G.: Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. J. Cell Biol., *31:* 319, 1966.
26. Holtzman, E. and Dominitz, R.: Lysosomes, Golgi apparatus and endoplasmic reticulum in secretion and protein uptake by adrenal medulla cells of the rat. J. Histochem., *16:* 320, 1968.
27. Jamieson, J. D. and Palade, G. E.: Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. J. Cell Biol., *34:* 577, 1967.
28. Mollenhauer, H. H. and Whaley, W. G.: An observation on the functioning of the Golgi apparatus. J. Cell Biol., *17:* 222, 1963.
29. Toda, K., Hori, Y. and Fitzpatrick, T. B.: The site of tyrosinase activity within the melanosome. Presented at the Society for Investigative Dermatology, Inc., Thirtieth Annual Meeting, New York, 1969.
30. Chian, L. T. Y. and Wilgram, G. F.: Tyrosinase inhibition: its role in suntanning and albinism. Science, *155:* 198, 1967.
31. Von Overbeck, L. and Philipp, E.: Elektronenmikroskopische Untersuchungen am Melanomalignon der Vulva. Z. Geburtsh. Gynaek., *168:* 167, 1968.

Zelickson et al.,(1972).

S. ZELICKSON,*
H. MOTTAZ,*
A. HUNTER*

35

AN ELECTRON MICROSCOPIC STUDY ON THE EFFECT OF ULTRAVIOLET IRRADIATION ON HUMAN SKIN: I. AUTOPHAGY AND MELANOSOME DEGRADATION IN MELANOCYTES†

INTRODUCTION

Direct visualization with the electron microscope probably offers the most curate method for the localization and identification of dendritic cells. On ultra-ructural examination, melanocytes, Langerhans cells, and indeterminate dendritic lls have been described in human epidermis (1). The indeterminate cell is aracterized by its location in the lower epidermis, its dendritic appearance, its k of desmosomes, and the absence of premelanosomes, melanosomes, Langerhans anules, or any other identifying granules within its cytoplasm (Fig. 1). Melano-me complexes have been found within keratinocytes, melanocytes, Langerhans lls, and melanophages. Acid phosphatase has been demonstrated within these mplexes and may play a role in the fate of these granules (2, 3). Recently it has en shown that complexes of melanin granules may be broken down within mel-nocytes and that this form of autophagy may be a major factor in the degradation melanosomes not discharged from the melanocyte (4, 5). To study these cells in quantitative manner, the number of each cell type as well as its volume ratio was lculated in normal and ultraviolet (UV) irradiated epidermis.

MATERIALS AND METHODS

The untanned forearm of Caucasian males was selected as a test site. The inimal erythema dose (MED) for the forearm was established using a hot quartz mp source (Burdick Co., Milton, Wisconsin, Type QA 450, peak transmission at

* Department of Dermatology, University of Minnesota Medical School, Minneapolis, Minnesota.

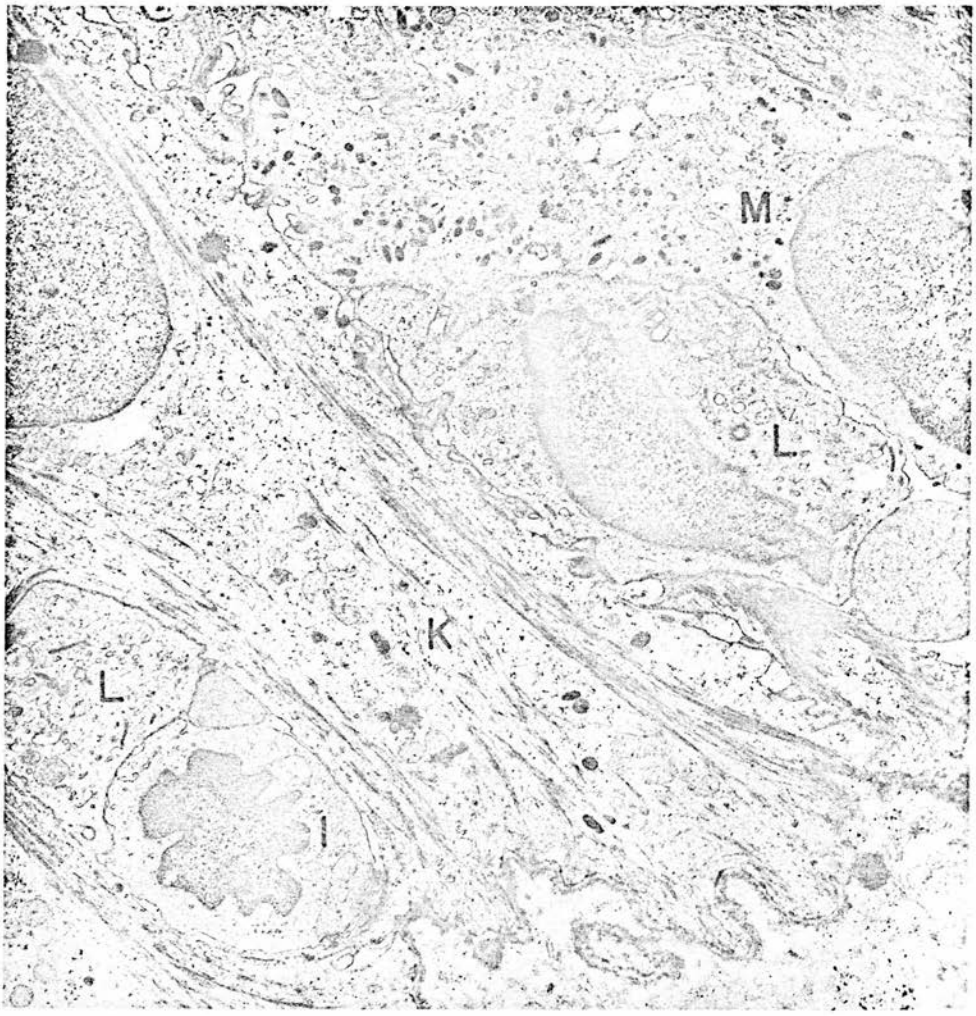


FIG. 1. *In addition to melanocytes (M) and Langerhans cells (L) an indeterminate (I) dendritic cell is present in human epidermis. K, keratinocyte.* × 9,240.

the 309 nm and 313 nm bands). The forearm was irradiated daily with 6 MED for 2 weeks. Tissue from the site was examined prior to irradiation and at 3 hours, 48 hours, and 2 weeks after irradiation. All tissue was fixed with osmium tetroxide, dehydrated in alcohol, and embedded in Epon according to standard procedures. Thin sections cut with an LKB ultrotoms were stained with uranyl acetate, followed by lead citrate, and viewed with an electron microscope.

Acid phosphatase was demonstrated by fixing the tissue pieces for 10 to 60 minutes at 4°C in 2 percent glutaraldehyde in 0.1 *M* cacodylate HCl buffer at pH 7.4. Sections were incubated for 45 minutes at 37°C in a modified Gomori medium

Following the incubation the tissue was postfixed in O_sO_4 and processed for electron microscopy.

The blocks were embedded in a random manner and 10 specimen blocks were selected from each site, also in a random manner, and sectioned accordingly. Five micrographs were taken of each of the 10 blocks; all photographs were taken in a random manner, giving a total of 50 prints for each site studied. The prints were then inserted under a regularly spaced grid of parallel lines, each line being 20 cm in length, giving a total of 100 cm for each print and a total of 50,000 mm for each site studied. Direct counting of the cells was also done in a random manner and in a manner not to include the same cell more than once. The stratum granulosum and stratum corneum were not included in the linear scanning.

RESULTS

Three hours after irradiation, melanosome complexes were numerous in the keratinocytes at the epidermo-dermal junction and usually capped the nucleus. Significantly, a number of active melanocytes were present in the upper dermis. The

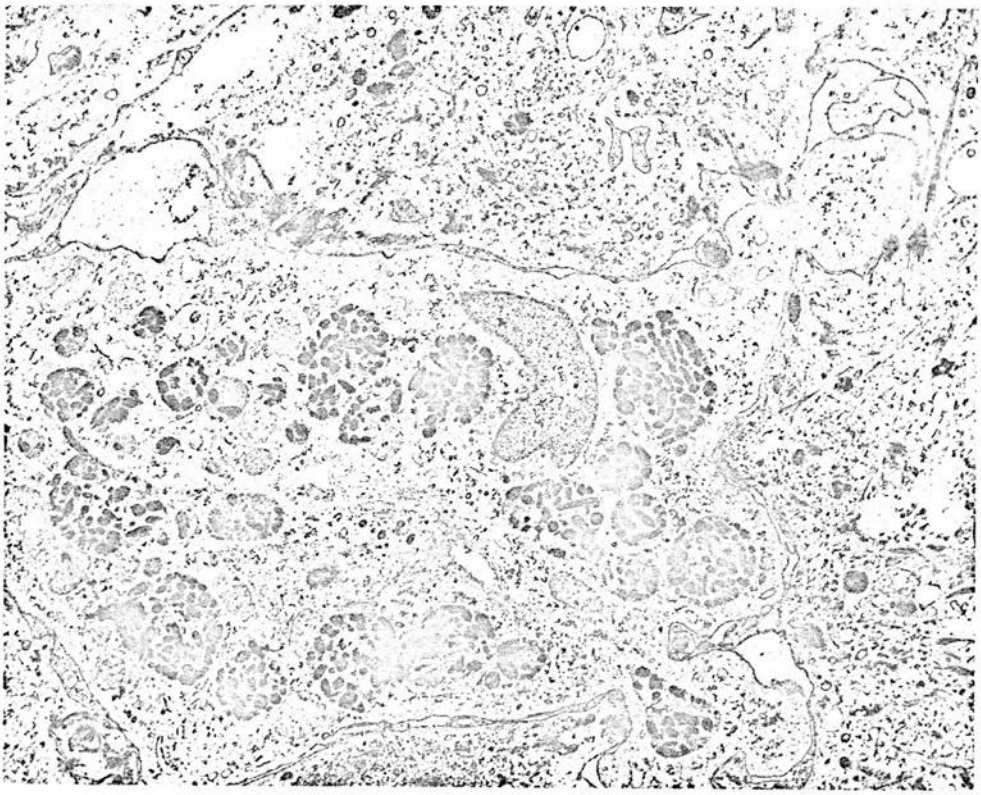


FIG. 2. *Numerous melanosome complexes were found within melanocytes following two daily exposures of 6 MED of UV irradiation.* × *12,650.*

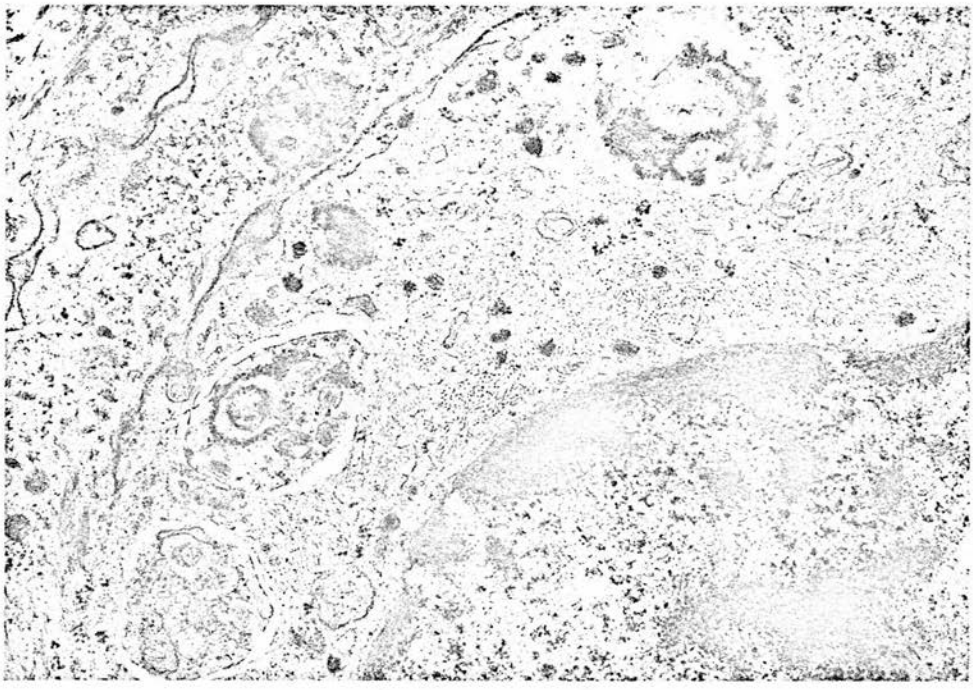


FIG. 3. *Mitochondria and portions of endoplasmic reticulum can be found within autophagic vacuoles in epidermal melanocytes.* × *25,740.*

TABLE 1
Quantitation of Epidermal Cells (Forearm)[a]

	VOLUME RATIO		DIRECT COUNT OF CELLS	
	NORMAL	UV-EXPOSED	NORMAL	UV-EXPOSED
Keratinocyte	93.80	93.27	152	169
Melanocyte	2.50	4.40	5	14
Langerhans cell	1.25	0.14	3	0
Indeterminate cell	0.95	0.23	3	0
Intercellular space	1.50	1.96	—	—

[a] *Following 2 weeks of daily exposure to 6 MED of UV light.*

endoplasmic reticulum and Golgi apparatus were well developed, and premelanosomes and melanosomes were numerous in these dermal melanocytes.

After two exposures to UV irradiation numerous melanosome complexes were located within melanocytes (Fig. 2) as well as keratinocytes. The melanocyte complexes consisted of groups of premelanosomes and melanosomes which were surrounded by a single unit membrane. Besides the latter, cellular organelles such as mitochondria and portions of endoplasmic reticulum were also found within these complexes (Fig. 3). Acid phosphatase could be demonstrated within the complexes. The number and size of the melanocyte complexes varied from one cell to the next. Often large empty vacuoles were also seen within the melanocytes, apparently the final stage after breakdown of the melanosome complexes.

After 2 weeks of continuous UV irradiation there was a marked increase in both the number and volume of melanocytes and, of most significance, an essential absence of Langerhans cells (Table 1).

DISCUSSION

The feasibility of applying the method of linear scan for volume ratio determinations to sections of tissue and the factors influencing the accuracy of these determinations have been reviewed by Carpenter and Lazarow (6). It has been demonstrated that the respective measure of distance traversed by a random linear scan through various tissue elements may be directly translated into a volume ratio, i.e., the distance traversed through component Y divided by the total tissue traversed times 100 equals the volume of component Y, expressed as a percentage of total tissue volume.

The autophagic capability of the melanocyte is strongly suggested by the grouping and segregation of premelanosomes, melanosomes, and cellular organelles into unit membrane limited cytoplasmic complexes. The empty vacuoles seen in the melanocytes which contained these complexes were all that remained following degradation of their contents. The latter is similar to the breakdown of the melanosome complexes reported in the Chediak-Higashi syndrome (4). The demonstration of acid phosphatase within these structures adds support to this concept. It is likely that melanosomes which do not leave the melanocyte may be broken down within the cell and that this process may serve as a pathway in the degradation of premelanosomes and melanosomes.

Direct counting, as well as linear scanning with the electron microscope, revealed a marked increase in active melanocytes and a sharp decrease in epidermal Langerhans cells following 2 weeks of daily UV irradiation. The increase in melanocytes may be accounted for by an increase in mitotic activity, although mitotic figures were not encountered in this study. Dermal melanocytes were not seen moving into the epidermis. Conversion of other dendritic cells to active melanocytes may account for some of the increase. The change in the number of indeterminate cells following UV irradiation was not great enough to account for the entire increase in melanocytes but could account for the early increase in the number of these

cells. Probably both of the aforementioned play a role in the increased number of melanocytes noted following UV irradiation.

Fan and associates (7) noted a decrease in the number of Langerhans cells following ultraviolet, X ray, and thorium X irradiation of guinea pigs and attempted to relate these observations to the simultaneous increase in the number of dopa-positive melanocytes. Breathnach et al. (8) also noted a decrease in the number of gold-positive cells and an increase in melanocyte number after application of thorium X to guinea pig skin. Our findings are in accord with the latter and with those of Fan et al. (7) and suggest some type of relationship between the increase in dopa-positive cells and the decrease in epidermal Langerhans cells.

The sharp drop in the number of epidermal Langerhans cells, which accompanied the increase in melanocyte number, is difficult to reconcile. A direct damaging effect of UV light on the Langerhans cell has been suggested (8), but the cells which were present did not show signs of damage. Quantitative investigations of vitiligo in sun-exposed sites, and more importantly, of vitiliginous skin deliberately exposed to UV irradiation, revealed an absence of melanocytes and if anything an increased number of unaltered Langerhans cells rather than a decrease in their number (1).

SUMMARY

It has been shown that the melanocyte has autophagic capabilities and can play an active role in melanosome degradation. An increase in melanocyte number and an essential absence of Langerhans and indeterminate cells were noted in skin from the human forearm following 2 weeks of daily UV irradiation.

REFERENCES

1. Zelickson, A. S., and J. H. Mottaz. 1968. Epidermal dendritic cells: a quantitative study. Arch. Derm., 98:652-658.
2. Hori, Y., K. Toda, M. Pathak, W. Clark, and T. Fitzpatrick. 1968. A fine-structure study of the human epidermal melanosome complex and its acid phosphatase activity. J. Ultrastruct. Res., 25:109.
3. Mishima, Y. 1966. Cellular and subcellular differentiation of melanin phagocytosis and synthesis by lysosomal and melanosomal activity. J. Invest. Derm., 46:70.
4. Zelickson, A. S., D. S. Windhorst, J. G. White, and R. A. Good. 1967. The Chediak-Higashi syndrome: formation of giant melanosomes and the basis of hypopigmentation. J. Invest. Derm., 49:575.
5. Kawamura, T., S. Ikeda, S. Mori, and H. Obata. 1966. Electron microscopic findings compatible with those of the lysosome (autophagic vacuole) revealed in the melanocytes in cases of conspicuous pigment blockade. J. Derm., 76:405.
6. Carpenter, A. M., and A. Lazarow. 1962. Component quantitation of tissue sections. J. Histochem. Cytochem., 10:329-340.
7. Fan, J., R. J. Schoenfeld, and R. Hunter. 1959. A study of the epidermal clear cells with special reference to their relationship to the cells of Langerhans. J. Invest. Derm., 32:445-449.
8. Breathnach, A. S., M. S. C. Birbeck, and J. D. Everall. 1963. Observation bearing on the relationship between Langerhans cells and melanocytes. Ann. N.Y. Acad. Sci., 100:223-237.

British Journal of Dermatology (1974) **90**, 481.

Clinical and Laboratory Investigations

Light and electron microscopic studies of physical injury to the skin
I. Suction

J.A.A.HUNTER, EVA McVITTIE AND J.S.COMAISH*

Department of Dermatology, University of Edinburgh, and
* Department of Dermatology, University of Newcastle

Accepted for publication 3 August 1973

SUMMARY

Suction applied to the skin causes almost immediate paranuclear vacuolization in keratinocytes but does not affect the dendritic cells. If suction is prolonged dermo-epidermal separation occurs at a level between the basal cell membrane and basal lamina.

Stress due to suction of the skin is not commonly encountered in everyday life. Horizontal shear forces are almost certainly those mainly involved in the production of friction damage, but nevertheless both pressure and suction forces are exerted on the foot in ordinary locomotion. Negative pressure may also be exerted on the skin of aviators (especially astronauts) and divers in certain circumstances. Under experimental conditions, suction has been used recently to evaluate dermo-epidermal adherence (Lowe & van der Leun, 1968) and to monitor the state of blistering diseases, such as porphyria cutanea tarda (Copeman, 1970). Some light microscopic and ultrastructural features of suction blisters have already been documented (Kiistala & Mustakallio, 1967; Copeman, 1970), but this paper deals primarily with intra-epidermal changes, which are in sharp contrast to those seen in frictional injury (Hunter, McVittie & Comaish, 1974).

MATERIAL AND METHODS

Twenty-five volunteers were studied, of whom six members of staff had no skin disease and the rest were patients with miscellaneous skin disorders (eczema, psoriasis, dermatitis herpetiformis and porphyria cutanea tarda). Blistering was produced on skin which was macroscopically normal, the flexor aspect of the forearm or the back of the hand being used. The suction chamber chosen was a modification of that described by Kiistala & Mustakallio (1967), and contained a perforated disc with seven apertures each of 5 mm diameter. The negative pressure was usually -150 mmHg but occasion-

This paper was read in part at the meeting of the Investigative Group of the British Association of Dermatology, 3 February 1973, London.

Reprint requests to Dr J.A.A.Hunter, Department of Dermatology, The Royal Infirmary, Edinburgh.

ally −300 mmHg. Biopsies, with and without local anaesthesia, were taken before and 1, 5, 15 and 25 min after the onset of suction, as well as immediately after the appearance of a blister (70–100 min).

Light microscopy

Part of the biopsy was immediately fixed in formalin for routine histology. Serial sections were stained with haematoxylin and eosin (H & E) or periodic acid-Schiff (PAS). Fresh cryostat sections were prepared for the demonstration of acid phosphatase activity using naphthol AS-TR phosphate and hexazonium pararosanilin according to the method of Barka & Anderson (1962).

Electron microscopy

Cubes of up to 1 mm^3 were immediately fixed in fresh 3% glutaraldehyde in cacodylate buffer pH 7·4 or in a half strength paraformaldehyde–glutaraldehyde fixative pH 7·4 (Karnovsky, 1965). Fixation was either at 4°C or at room temperature for 5–24 h. Some specimens were fixed in 3% glutaraldehyde containing 1% lanthanum nitrate at 4°C for 3 days (Hashimoto, 1970). After thorough buffer washes the specimens were postfixed in buffered 1% osmic acid. Before routine dehydration, most blocks were precontrasted with 0·5% aqueous uranyl acetate (Farquhar & Palade, 1965). The blocks were embedded in Araldite and ultrathin sections were cut with an LKB III Ultramicrotome and contrasted with uranyl acetate and lead citrate. They were examined using an AEI EM6 electron microscope.

Altogether, twenty-five biopsies were processed and ten of these were examined electron microscopically. The results recorded are based on the study of numerous light microscopic serial sections and over 200 electron micrographs.

RESULTS

The light and electron microscopic appearances were similar in all the subjects studied and are best grouped as a whole.

Light microscopy

After less than 1 min of suction, appreciable changes were noted compared with control biopsies obtained without suction. There was slight oedema of the superficial dermis, and paranuclear vacuoles were seen in increasing numbers as the suction time increased; by 25 min nearly all the epidermal cells were affected (Fig. 1), though there was no suggestion of dermo-epidermal separation. Some of the cells extending down follicular walls contained these distinctive vacuoles. They were invariably paranuclear, most often circular or ovoid in cross section, and frequently caused a crescentic deformation of the nuclear outline. The spatial relationship with the nucleus was variable; some vacuoles were superficial and others were deep to or beside the nucleus. Even after 25 min of suction there was no evidence of spongiosis (extracellular oedema). Biopsies taken just after blister formation revealed dermo-epidermal separation, and PAS-stained sections showed that the positive staining basal lamina region remained on the topmost dermis (Fig. 2), forming the blister floor. The cleavage line was frequently clear cut, but sometimes basal cells or dendritic cells (Fig. 2) remained on the floor of the cavity. The roof of the blister was, therefore, formed by the total thickness of the epidermis. It usually, though not invariably, contained numerous vacuolated cells and at this late stage spongiosis could sometimes be seen (Fig. 4). Sections processed to show acid phosphatase activity showed no obvious abnormality in the distribution of the red reaction product.

Electron microscopy

Keratinocytes. The vacuoles in these cells were quite characteristic and were seen in a few cells as early as 3 min after the onset of suction. They were bounded by a membrane (Fig. 3), particularly

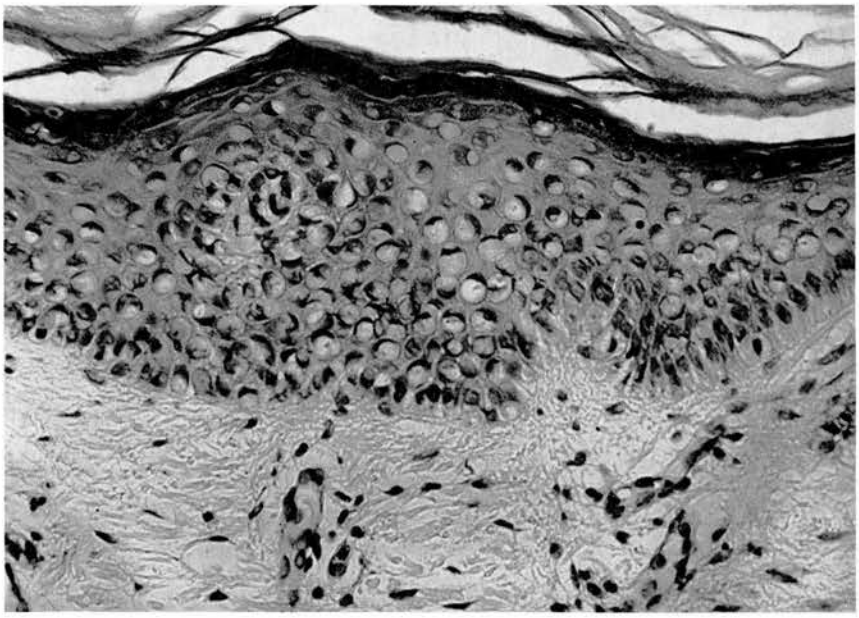


FIGURE 1. 25 min. −150 mmHg. Nearly all the keratinocytes contain large paranuclear vacuoles, and there is no sign of dermo-epidermal separation (H & E, ×270).

FIGURE 2. 135 min. −150 mmHg. An early blister forming by dermo-epidermal separation. The PAS positive staining basal lamina region remains on the floor (large arrows). A probable melanocyte (small arrow) has also been left behind on the floor (PAS, ×720).

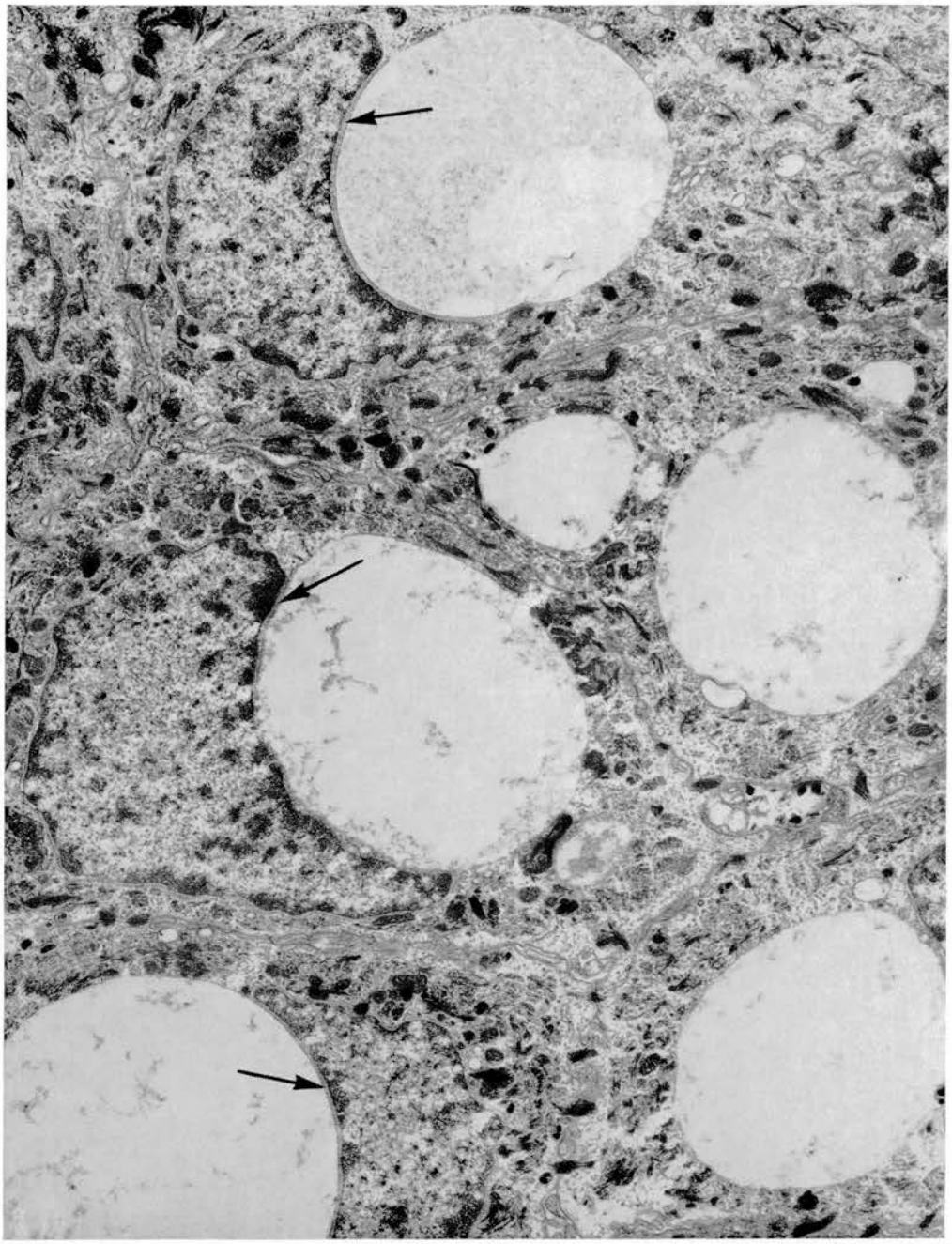


FIGURE 3. 25 min. −150 mmHg. Preblister. Paranuclear vacuoles are prominent in this area. They deform the nuclei and contain varying amounts of granular material. There is a small rim of cytoplasm between the outer nuclear membrane and the vacuole membrane (arrows) (× 12,250).

during the earlier stages of their development, and in some instances this membrane could be resolved into a trilaminar structure. They rapidly achieved a diameter of up to 6 μm, and when they reached this size they were invariably seen next to the nucleus, which they deformed into a half-moon shape (Fig. 3). They were not within the perinuclear space, which appeared to be normal (Figs. 3 and 6). Quite often a thin but distinct rim of cytoplasm could be seen between the outer nuclear membrane and the vacuolar membrane (Fig. 3). They were often filled with a fine granular material that tended to accumulate towards the periphery of the larger vacuoles (Fig. 3). With increasing suction, the vacuoles increased in size and their limiting membranes began to show breaks; their outlines became

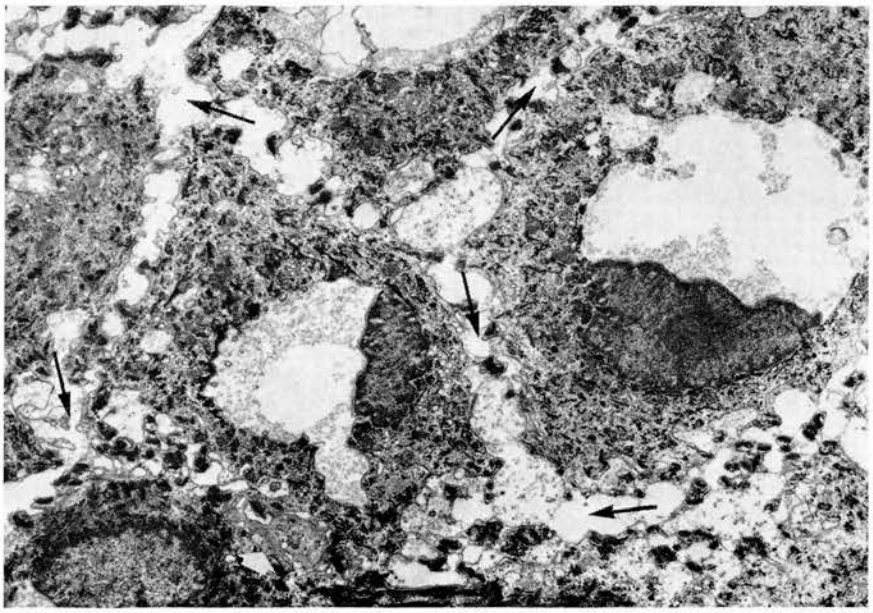


FIGURE 4. 90 min. −150 mmHg. Edge of blister roof. The outline of the perinuclear vacuoles is irregular and there is considerable intercellular oedema (long arrows). The broad white arrow points to a dendritic process of a Langerhans' cell (× 6000).

much less regular (Figs. 4, 6 and 7), and melanosomes (Fig. 8), melanosome complexes (Fig. 7) and membranous remnants (Fig. 6) could be seen within their cavity. At the blistering time, smaller membrane-bound vacuoles (diameter 80–160 nm) could also be seen near the edges of the ruptured paranuclear vacuoles (Figs. 6 and 7). At this late stage, the mitochondia showed degenerative changes. The distribution of the tonofibrils appeared relatively normal, though sometimes they were compressed by the larger vacuoles.

Dendritic cells. Significantly, neither melanocytes nor Langerhans' cells were seen to contain similar paranuclear vacuoles. Over twenty dendritic cells were seen to be surrounded by keratinocytes containing vacuoles, though they themselves were not affected (Fig. 5).

Intercellular space. This appeared normal at 25 min after the onset of suction, even though most cells contained paranuclear vacuoles. At the blistering time, however, a variable amount of intercellular oedema was seen, particularly at the blister edge, and in some places the distended space contained a fine granular material similar to that in the paranuclear vacuoles (Fig. 4).

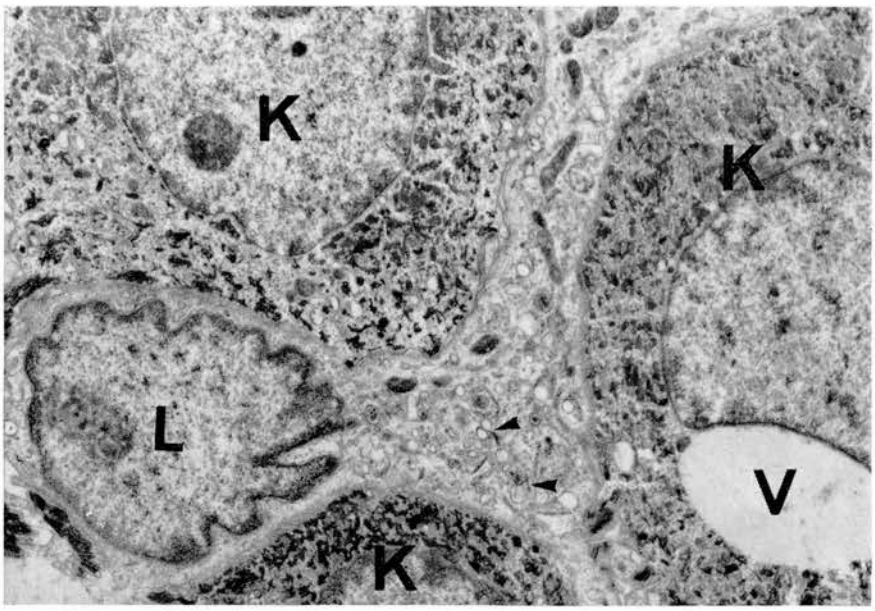


FIGURE 5. 90 min. −150 mmHg. Blister roof. A Langerhans' cell (L) with characteristic granules (small arrows) and dendrites does not contain a paranuclear vacuole. One (V) is seen in a neighbouring keratinocyte (K) (×9600).

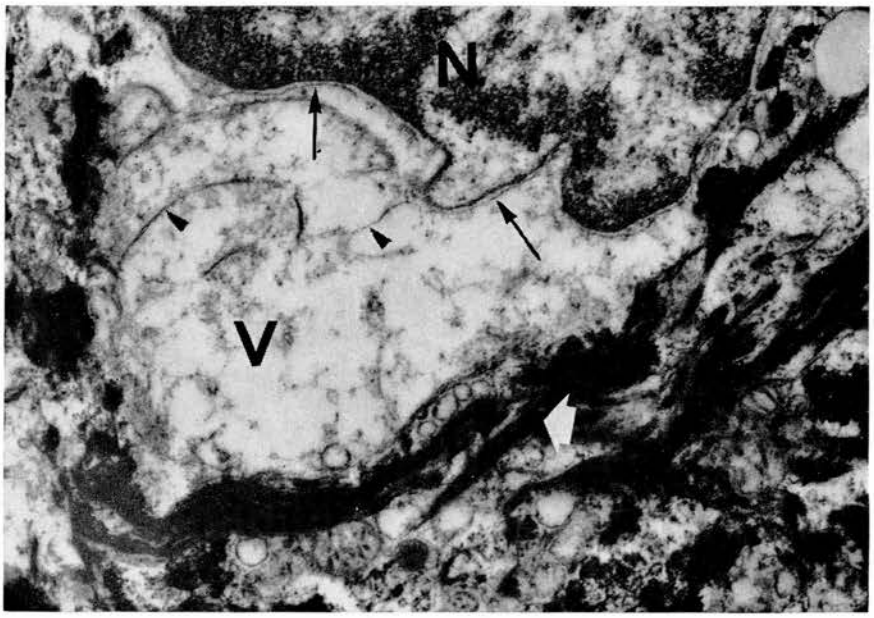


FIGURE 6. 110 min. −150 mmHg. Blister roof. Keratinocyte. The limiting membrane of the paranuclear vacuole contains a granular material and membranous remnants (short arrows). The long arrows point to the intact outer nuclear membrane. N, nucleus; broad white arrow, tonofilaments (×27,900).

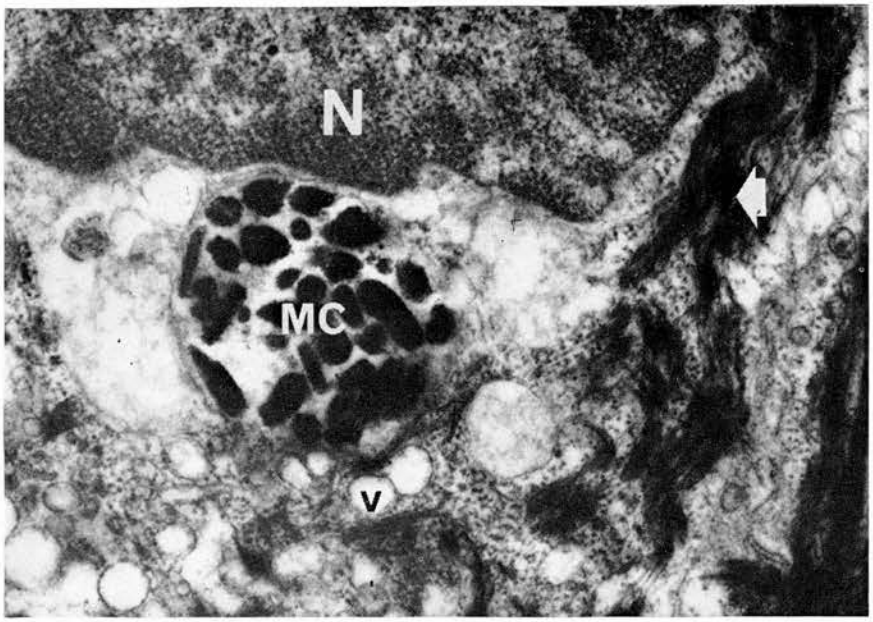


FIGURE 7. 110 min. −150 mmHg. Blister roof. Keratinocyte. The limiting membrane of the paranuclear vacuole has ruptured and a membrane bound melanosome complex (MC) lies in the remains of the vacuole. Smaller vacuoles (V) are seen at the edge of the disintegrating vacuole. Broad white arrow, tonofilaments (×37,100).

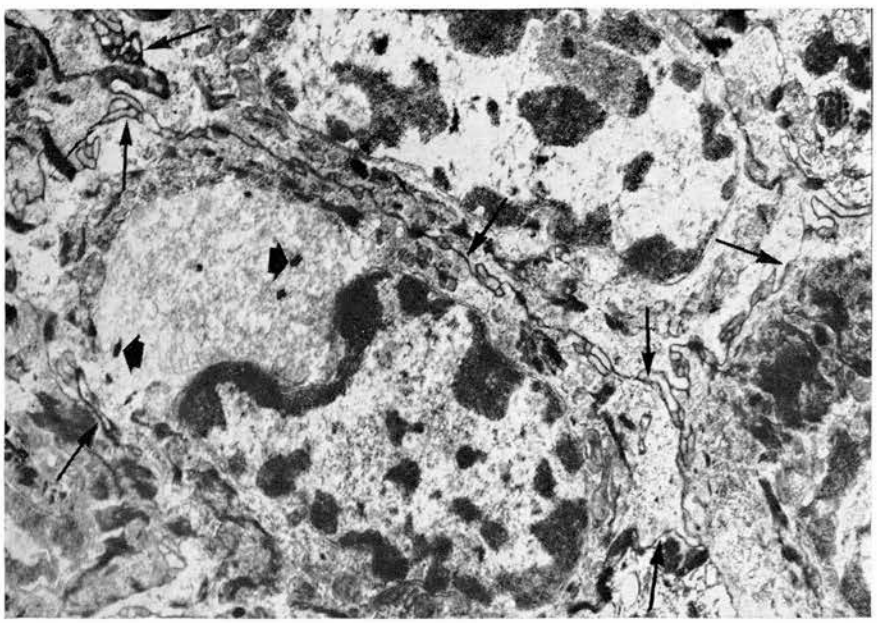


FIGURE 8. 95 min. −150 mmHg. Blister roof. Lanthanum impregnation. The lanthanum (long arrows) outlines the intercellular space around a keratinocyte containing a paranuclear vacuole, but there is no evidence of tracer within the vacuole. Broad arrows, single melanosomes in vacuole (×11,400).

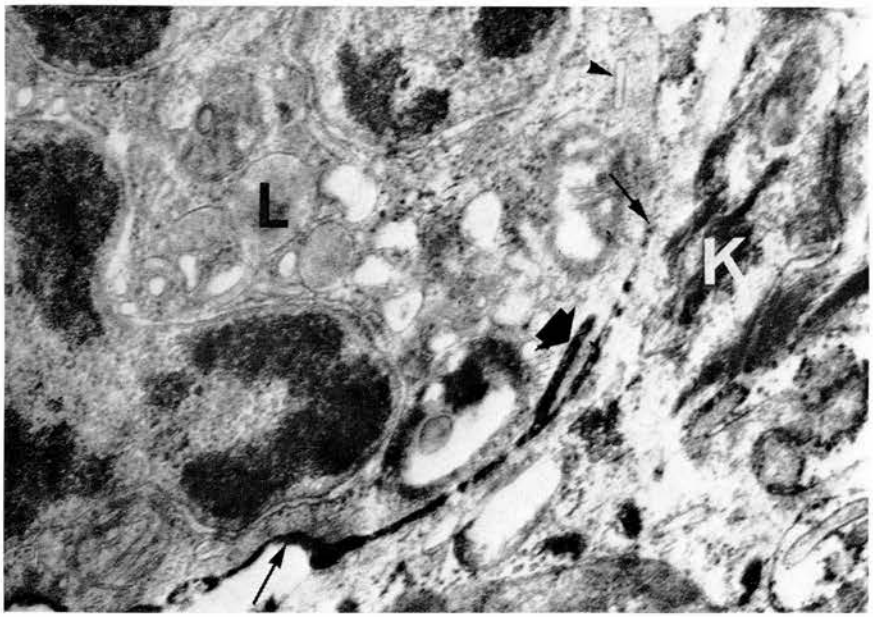


FIGURE 9. 95 min. −150 mmHg. Blister roof. Lanthanum impregnation. The lanthanum (long arrows) stains the intercellular space and a Langerhans' cell granule (broad arrow) attached to the wall of the Langerhans' cell (L). Small arrow points to an unstained granule. K, keratinocyte (×24,900).

Basal region. The blister was formed by dermo-epidermal separation. Cleavage was most often between the base of the basal cell and the basal lamina, so that the latter usually remained on the floor of the blister. However, fragments of the basal lamina were rarely seen attached to the base of the basal cells in the roof, and sometimes the cleft appeared less neatly through the base of some basal cells. Keratinocytes and melanocytes were occasionally seen in the blister fluid or lying on the floor of the blister.

Lanthanum impregnation. The intercellular space showed a normal appearance when impregnated with lanthanum (Figs. 8 and 9), and the tracer was never seen in the paranuclear vacuoles of keratinocytes (Fig. 8). The lanthanum did, however, stain granules attached to Langerhans' cell walls (Fig. 9).

DISCUSSION

Kiistala & Mustakallio (1967) concentrated on changes at the dermo-epidermal junction when assessing the ultrastructural effects of suction. In this area our findings are in broad agreement with theirs. It is interesting to note the similarity of cold-induced blisters, especially with regard to the site of ultimate blister formation, though in these the epidermal cells are grossly well-preserved (Pearson, 1965). Kiistala & Mustakallio also mentioned that a few keratinocytes contained vacuoles which caused a peculiar crescentic displacement of an otherwise normal-looking nucleus and considered that the vacuoles were situated between the inner and outer nuclear membranes. We would agree with Copeman (1970), who commented that the vacuoles and nuclear displacement were a most noticeable feature of suction and could be seen constantly.

The nature and mechanism of formation of the paranuclear vacuoles present interesting problems. Similar vacuoles have been noted in human keratinocytes in dermographic weals (Cauna, Macy & Cralley, 1970), and have been produced experimentally by ultraviolet irradiation (Nix, 1967), by intradermal injection of hypertonic solutions of sodium chloride and of dextrose (Hönigsmann & Wolff, 1973), by suction (Copeman, 1970), and by the topical application of vitamin A to the human skin (Plewig, Wolff & Braun-Falco, 1971). Wolff & Hönigsmann (1973) believed that the vacuoles seen in this study are identical to those produced by the intracutaneous injection of hypertonic solutions into guinea-pig skin. They have carried out extensive studies to determine their nature and mechanism of formation. Using tracers such as colloidal silver and horse-radish peroxidase in the injected solution, they were able to show staining of both the intercellular space and the paranuclear vacuoles. The vacuoles were stained also with osmium soaking (a technique which outlines the endoplasmic reticulum) and did not contain ruthenium red or nucleoside triphosphatase activity, suggesting that they were not formed by infolding of the cell membrane. They concluded that there was a canalicular system of interconnected cisternal channels within keratinocytes, which formed a direct communication between the extracellular and the perinuclear spaces, and that it could unfold under certain experimental and pathological conditions (Hönigsmann & Wolff, 1973). In this study, the development of the vacuoles was too quick for it to be possible to consider endocytic mechanisms for the uptake of the fluid. The rapidity of their formation suggests also that fluids are sucked from the intercellular space into pre-existing cavities along communications of endoplasmic reticulum, as suggested by Hönigsmann & Wolff.

The precise role of lanthanum in staining the cell surface is uncertain. Doggenweiler & Frenk (1965) considered that it stained lipids of the cell surface, while Behnke (1968) and Shea (1971) felt that its staining was specific for the demonstration of acid mucosubstances. Its delineation of the extracellular space seems to be due to non-specific precipitation (Revel & Karnovsky, 1967). It has been used to outline the extracellular space in human epidermis (Wolff & Schreiner, 1968), and to show continuity between the extracellular space and granules attached to the walls of Langerhans' cells (Hashimoto, 1970). In this study, it was at first surprising to note that there was no sign of lanthanum penetration into the paranuclear vacuoles within keratinocytes. The most likely explanation of this would seem to be that a communication, like that envisaged by Hönigsmann & Wolff, is patent only during the period of suction. As this communication cannot be penetrated by lanthanum in normal skin (Wolff & Schreiner, 1968), Hönigsmann & Wolff have tentatively suggested the presence of a valve or sluice-like mechanism which opens only under certain circumstances, e.g. when in contact with hypertonic solutions or an increased flow of intercellular fluid. If this were so, then the lanthanum, which was used only during fixation of the biopsy specimen, would not be expected to gain access to the postulated canalicular system of communication.

Although ultrathin serial sections were not made, the fact that paranuclear vacuoles were not seen in melanocytes or Langerhans' cells seemed significant. Examination of numerous sections revealed no affected dendritic cells, even though neighbouring keratinocytes contained large paranuclear vacuoles. It should also be pointed out that neither Cauna *et al.* (1970) nor Hönigsmann & Wolff (1973) mentioned such vacuoles in epidermal dendritic cells, in spite of their obvious presence in keratinocytes. There are two likely explanations. First, it is possible that communications between the extracellular space and the system of endoplasmic reticulum, demonstrated in keratinocytes (Hönigsmann & Wolff, 1973), do not exist in dendritic cells. Secondly, it is conceivable that keratinocytes, because of their intercellular connections (desmosomes, intertwining villous processes and intercellular cement substance), are more prone to the effects of suction than dendritic cells, which merely move as a whole in the direction of the suction force.

ACKNOWLEDGMENT

This study was supported in part by a grant from the Scottish Hospital Endowment Research Trust (HERT 332).

REFERENCES

BARKA, T. & ANDERSON, P.J. (1962) Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. *Journal of Histochemistry and Cytochemistry*, **10**, 741.

BEHNKE, O. (1968) Electron microscopic observations on the surface coating of human blood platelets. *Journal of Ultrastructure Research*, **24**, 51.

CAUNA, N., MACY, I.L. & CRALLEY, A. (1970) The fine morphology of the human skin in dermographism. *Journal of Allergy*, **45**, 266.

COPEMAN, P.W.M. (1970) Porphyria: successful treatment by alkalinisation of urine with sodium bicarbonate assessed by experimental suction blister apparatus. *British Journal of Dermatology*, **82**, 385.

DOGGENWEILER, C.F. & FRENK, S. (1965) Staining properties of lanthanum on cell membranes. *Proceedings of the National Academy of Sciences of the United States of America*, **53**, 425.

FARQUHAR, M.G. & PALADE, G.E. (1965) Cell junctions in amphibian skin. *Journal of Cell Biology*, **26**, 263.

HASHIMOTO, K. (1970) Lanthanum staining of Langerhans' cell. *Archives of Dermatology*, **102**, 280.

HÖNIGSMANN, H. & WOLFF, K. (1973) Continuity of intercellular space and endoplasmic reticulum of keratinocytes. *Journal of Experimental Cell Research*, **80**, 191.

HUNTER, J.A.A., MCVITTIE, E. & COMAISH, J.S. (1974) Light and electron microscopic studies of physical injury to the skin. II. Friction. *British Journal of Dermatology*, **90**, 491.

KARNOVSKY, M.J. (1965) A formaldehyde–glutaraldehyde fixative of high osmolarity for use in electron microscopy. *Journal of Cell Biology*, **27**, 137A.

KIISTALA, U. & MUSTAKALLIO, K.K. (1967) Dermo-epidermal separation with suction. Electron microscopic and histochemical study of initial events of blistering on human skin. *Journal of Investigative Dermatology*, **48**, 466.

LOWE, L.B. & VAN DER LEUN, J.C. (1968) Suction blisters and dermo-epidermal adherence. *Journal of Investigative Dermatology*, **50**, 308.

NIX, T.E. (1967) Ultraviolet-induced changes in epidermis. In: *Ultrastructure of Normal and Abnormal Skin* (Ed. by A.S.Zelickson), p. 304. Lea and Febiger, Philadelphia.

PEARSON, R.W. (1965) Response of human epidermis to graded thermal stress: a morphologic comparison of burns, cold-induced blisters, and pemphigus vulgaris. *Archives of Environmental Health*, **11**, 498.

PLEWIG, G., WOLFF, H. & BRAUN-FALCO, O. (1971) Lokalbehandlung normaler und pathologischer menschlicher Haut mit Vitamin a-Säure. Klinische, histologische und elektronenmikroskopische Untersuchungen. *Archiv für klinische und Experimentelle Dermatologie*, **239**, 390.

REVEL, J.P. & KARNOVSKY, M.J. (1967) Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *Journal of Cell Biology*, **33**, C7.

SHEA, M.S. (1971) Lanthanum staining of the surface coat of cells. Its enhancement by the use of fixatives containing alcian blue or cetylpyridinium chloride. *Journal of Cell Biology*, **51**, 611.

WOLFF, K. & HÖNIGSMANN, H. (1973) Personal communication.

WOLFF, K. & SCHREINER, E. (1968) An electron microscopic study of the extraneous coat of keratinocytes and the intercellular space of the epidermis. *Journal of Investigative Dermatology*, **51**, 418.

British Journal of Dermatology (1974) **90,** 491.

Light and electron microscopic studies of physical injury to the skin
II. Friction

J.A.A.HUNTER, EVA McVITTIE AND J.S.COMAISH*

Department of Dermatology, University of Edinburgh, and
* Department of Dermatology, University of Newcastle

Accepted for publication 3 August 1973

SUMMARY

Frictional injury to the skin causes inter- and intra-cellular oedema of the epidermis, and if severe produces membranous rupture and formation of an intra-epidermal blister. The appearances are consistent with direct physical damage to the cells.

Both for gripping and for moving, man cannot function without friction between him and his environment. However, if this stress is prolonged or unusually severe, pathological changes occur, culminating in blisters and erosions. Human epidermis subjected to frictional stress has been studied light microscopically (Naylor, 1955; Fukuyama & Cortese, 1968), and autoradiographically (Epstein, Fukuyama & Cortese, 1969), but to date no electron microscopic studies have been published. This paper correlates the light microscopic changes with electron microscopic appearances and contrasts both with those produced by suction (Hunter, McVittie & Comaish, 1974).

MATERIAL AND METHODS

Six volunteers with ages ranging from 20 to 78 years were studied. Four had miscellaneous skin diseases (psoriasis, three, and tinea pedis, one). Macroscopically normal skin on the dorsal surface of the forearm was chosen for the experiments. The apparatus used was similar to that described by Naylor (1955), and consisted of a machine which rubbed skin with a reciprocating action at measurable speeds and forces until a blister or its immediate consequence, an erosion, was produced. The head was hemispherical and made of stainless steel. A description of the machine and its operation has already been published (Comaish, 1973). The friction force varied from 1·9 to 3·9 Newtons† and blistering occurred at times ranging from 5 to 14 min. Two of the subjects agreed to having

This paper was read in part at the meeting of the Investigative Group of the British Association of Dermatology, 3 February 1973, London.

Reprint requests to: Dr J.A.A.Hunter, Department of Dermatology, The Royal Infirmary, Edinburgh.

† 1 Newton (N) causes an acceleration of unity (1 metre per second per second) to a 1 kilogram mass.

biopsies taken from four different sites, allowing sequential changes to be studied up to the time of an erosion (e.g. biopsies at 1, 5, 10 and 13 min after the onset of friction).

The light and electron microscopic techniques were as reported in our study on suction blisters (Hunter *et al.*, 1974).

Altogether fourteen biopsies were taken. Ten were prepared for light microscopy and were serial sectioned, and the remaining four (two taken just after blister formation and two approximately midway between the onset of friction and expected blister time) were processed for electron microscopy.

RESULTS

There was no significant difference noted between different subjects, and the results are therefore grouped as a whole. They are most conveniently summarized by describing changes seen at the time of blistering (e.g. 14 min) and those seen much earlier (e.g. 5 min), where the biopsy was taken from intact but slightly erythematous skin. The changes in the latter (hereafter described as 'early' changes) were more subtle and could often be traced only by searching through numerous serial sections with light microscopy, and by carefully checking thick sections embedded in Araldite before cutting ultrathin sections for electron microscopy.

Light microscopy

Early changes. The earliest changes were noted in the upper Malpighian layer (Fig. 1), whilst the overlying horny and granular cell layers were relatively unaffected. The changes were quite frequently noted adjacent to hair follicles and sweat duct orifices, and consisted of a localized area of pale and washed out eosinophilic change in the haematoxylin and eosin sections (arrow in Fig. 1). The cells showed marked intracellular oedema, their margins were indistinct, and there was some spongiosis. Small vesicles could be seen when the changes were more developed (Fig. 1). The superficial dermis showed only mild oedema.

Late changes. Blistering occurred intra-epidermally at the level of the upper Malpighian layer (Fig. 2). Many of the neighbouring epidermal cells in the floor and to the side of the blister showed similar changes to those seen in the early stages and described above. These sometimes extended to the level of the basal cells (broad arrow, Fig. 2), but the dermo-epidermal junction remained intact. However, below the blister the basal lamina region stained less intensely with PAS (Fig. 2). The superficial dermis still showed only mild oedema.

Electron microscopy

Early changes (Figs. 3, 4, 7 and 8). Figs. 3, 4 and 7 are representative of the early changes seen in areas such as that arrowed in Fig. 1. There was obvious intracellular oedema (broad arrows, Fig. 3), which was most noticeable at the cell periphery, and when severe produced a granular appearance in an area devoid of organelles (Fig. 4). As a result of this, the tonofilaments sometimes appeared clumped, resembling necklaces around the nuclei (Fig. 3). In places, small vacuoles (approximately 0·8 μm in diameter) appeared in the oedematous periphery of keratinocytes, and the membranes of these cells were often ruptured (V_1, Fig. 3), allowing their granular contents to spill into small dilatations of the extracellular space (V_2 and V_3, Fig. 3). The few dendritic cells seen appeared to be relatively unaffected.

Late changes (Figs. 5 and 6). Similar though more pronounced changes were seen around established blisters. Figs. 5 and 6 are representative of changes seen in an area adjacent to the broad arrow

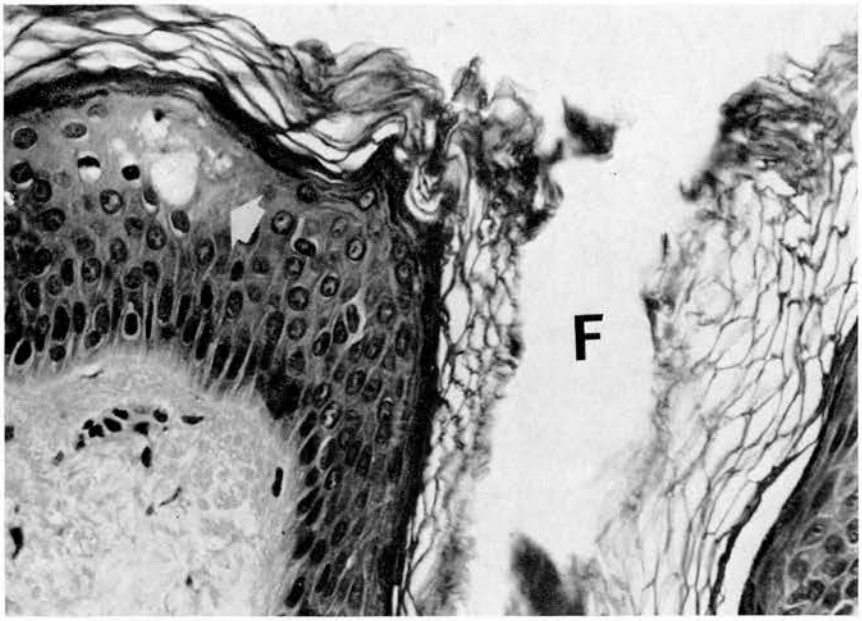


FIGURE 1. Early changes (5 min rubbing, 2·6 N) are indicated by the broad arrow and are seen just deep to the granular cell layer. F, follicle (H & E ×420).

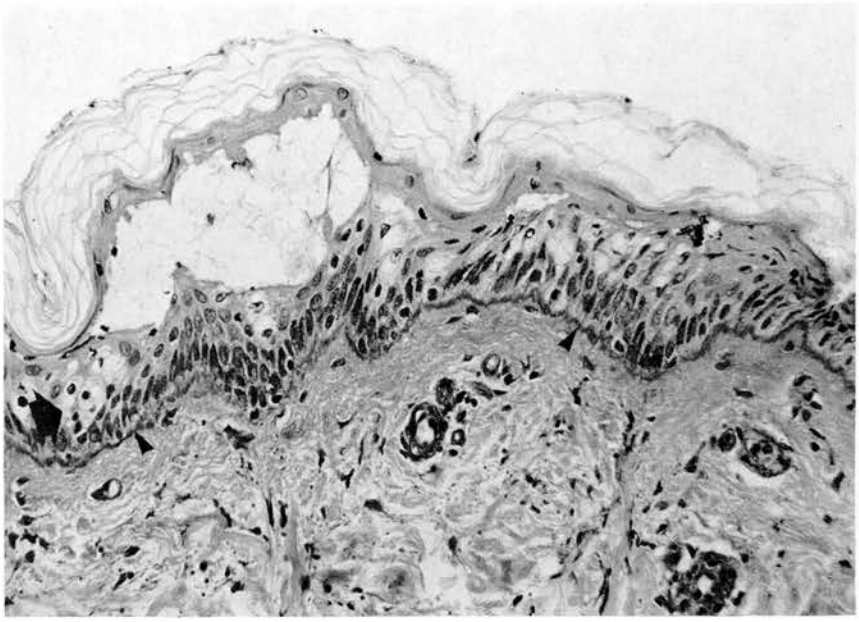


FIGURE 2. Late changes (14 min rubbing, 2·0 N) show an established intra-epidermal blister in the upper Malpighian layer. Broad arrow points to cells in base which show marked intracellular oedema. The basal lamina (small arrows) shows some lack of definition below the blister but is otherwise intact (PAS, ×270).

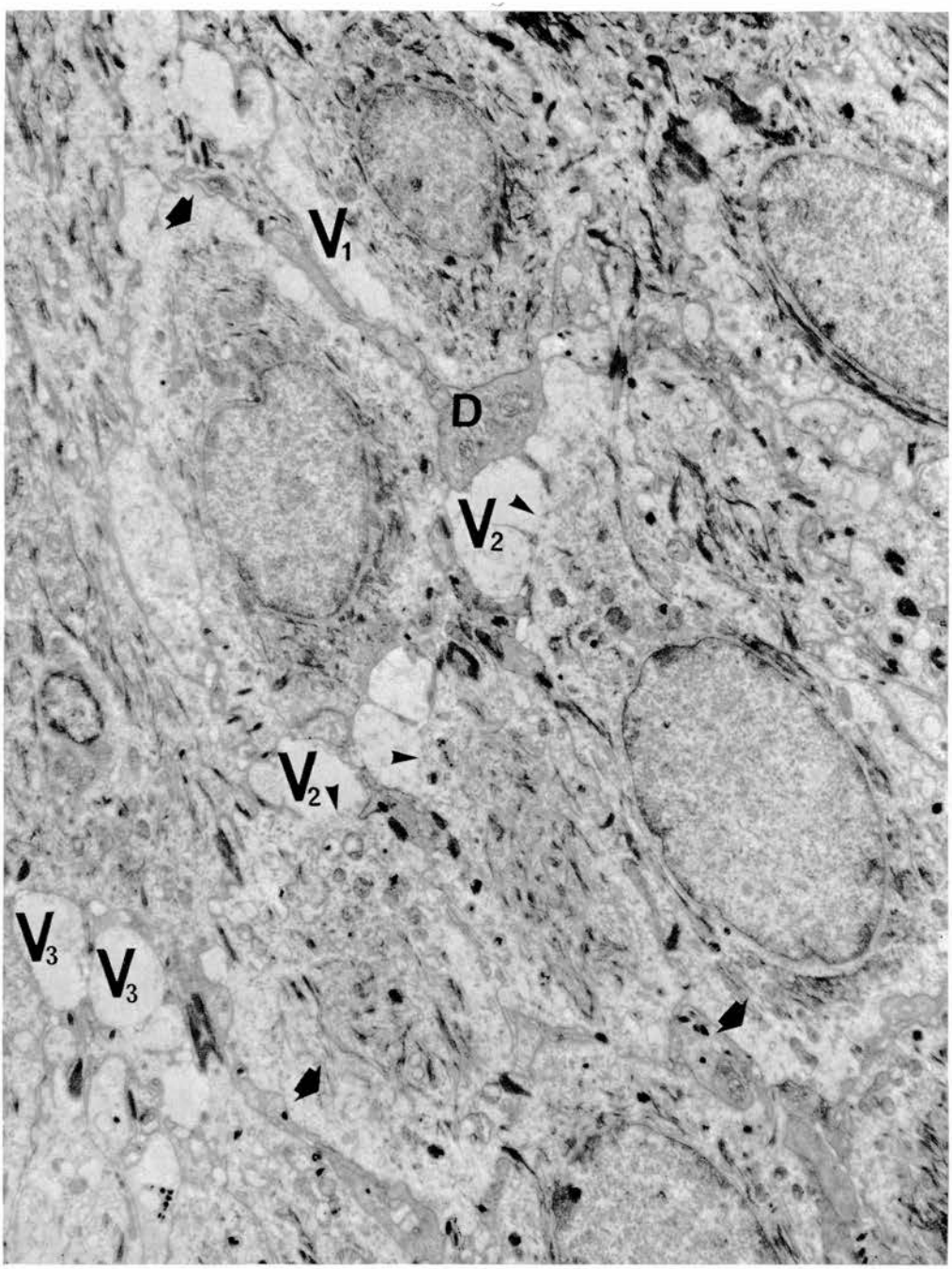


FIGURE 3. Early changes (5 min rubbing) seen in an area similar to that arrowed in Fig. 1. The keratinocytes show marked intracellular oedema (broad arrows) and the cell peripheries are relatively devoid of organelles. Small arrows point to places where the membranes are ruptured. V_1, membrane bound vacuoles in the periphery of the cell; V_2, vacuoles in extra-cellular space containing granular material adjacent to ruptured membranes; V_3, extra-cellular space dilatations; D, dendritic cell (×7000).

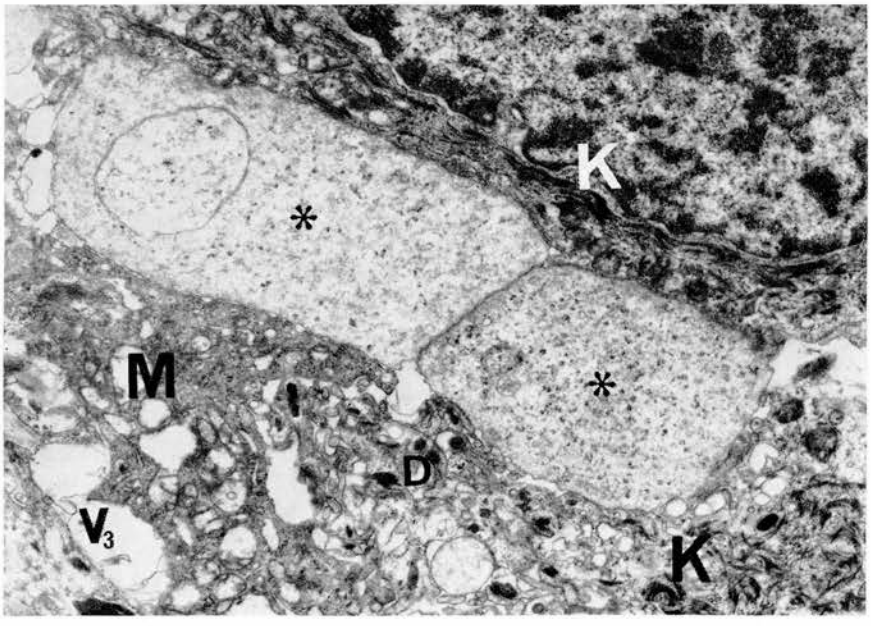


FIGURE 4. Early changes (5 min rubbing). Degenerative remnants of cells (*) contain a granular material and are free of organelles. K, keratinocyte; M, melanocyte; D, desmosome; V_3, extracellular space dilatation (× 11,700).

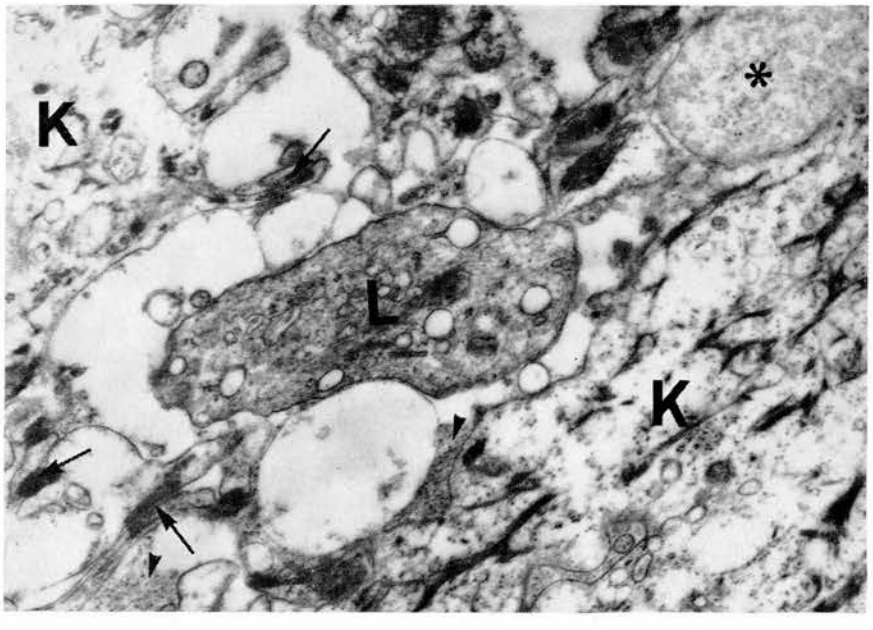


FIGURE 5. Late changes (6·5 min rubbing) at the edge of an erosion seen in an area similar to that indicated in Fig. 2. There are numerous dilatations of the extracellular space and some contain a granular material (small arrows) similar to that seen in degenerative cells (*) and oedematous keratinocytes (K). The process of a Langerhans' cell (L) appears unaffected. Large arrows point to intact desmosomes (× 20,700).

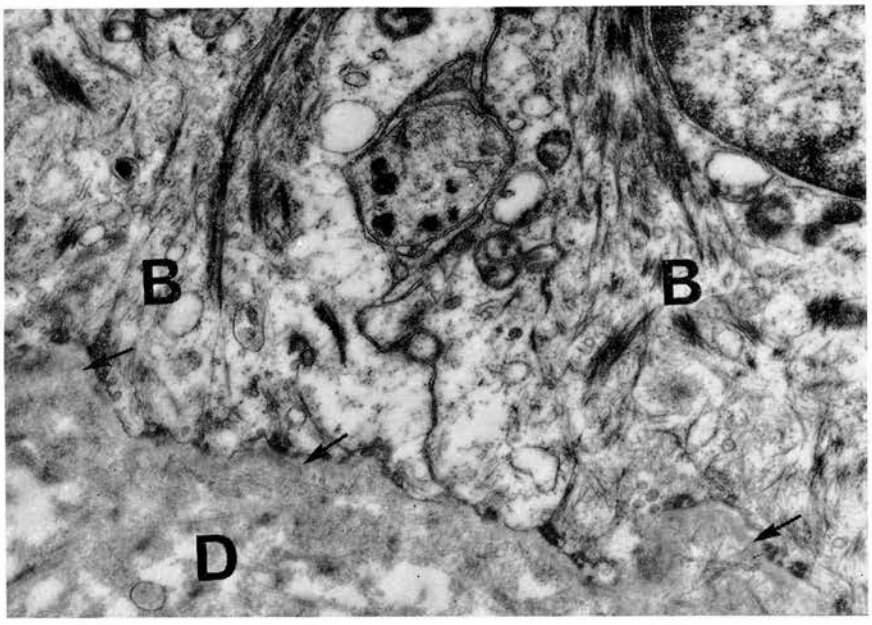


FIGURE 6. Late changes (6·5 min rubbing) seen in basal cells (B) in the floor of an erosion. The basal cells show marked oedema. The basal lamina (arrows), although indistinct in places, is essentially intact. D, dermis (× 17,700).

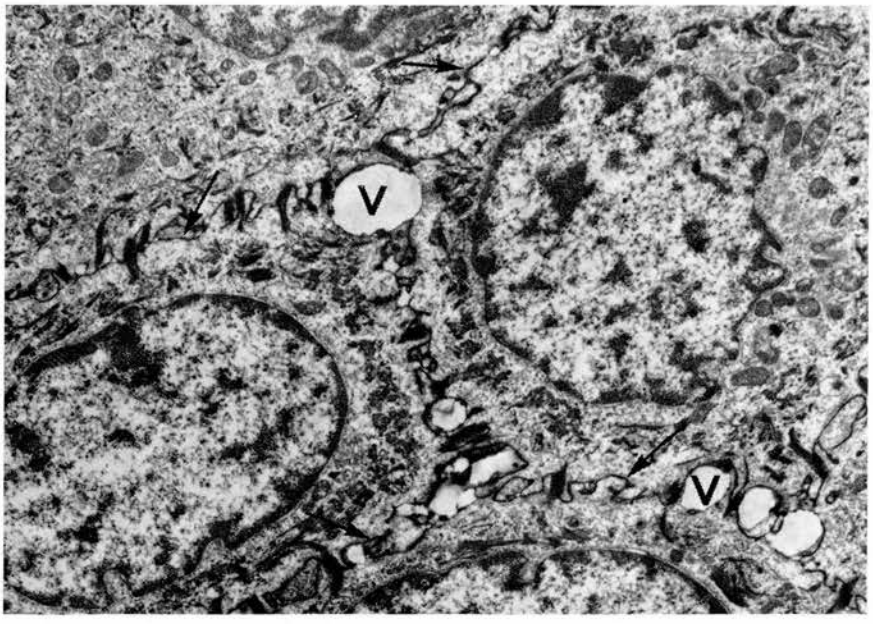


FIGURE 7. Early changes (5 min rubbing). Lanthanum preparation. Lanthanum (arrows) outlines the extracellular space (arrows) and the localized dilatations (V) (× 7200).

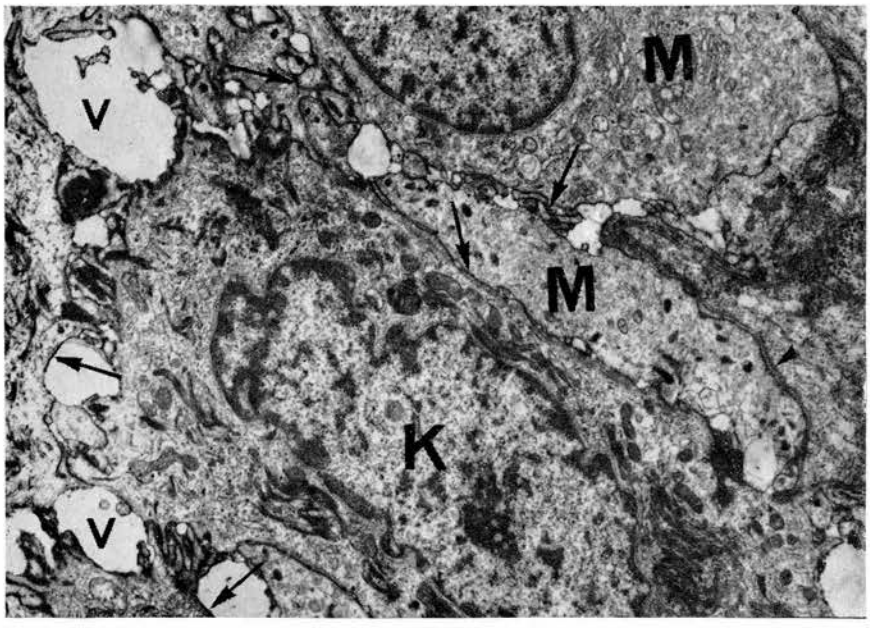


FIGURE 8. Early changes (5 min rubbing) are also seen in the extracellular space near the dermo-epidermal junction. Lanthanum preparation. Lanthanum (arrows) outlines the space and its localized dilatations (V). M, melanocyte; K, keratinocyte; small arrows point to basal lamina (×7800).

in Fig. 2. The intracellular oedema, again most marked at the periphery of keratinocytes, was increased and the extracellular space showed numerous localized dilatations which frequently contained a granular material (short arrows, Fig. 5). The cellular organelles were damaged and distorted, and on a very few occasions vacuoles (approximate diameter 0·3 μm) were seen beside keratinocyte nuclei. They were much smaller than those seen in the previous suction experiments and never appeared to cause nuclear deformation. Some desmosomal attachments were still evident (long arrows, Fig. 5), though membranous rupture and intracellular oedema often made identification of the cell borders difficult. The large intra-epidermal blisters contained in places a granular material devoid of organelles.

Lanthanum staining. Sections from lanthanum treated blocks showed the tracer outlining both the extracellular space and its localized dilatations (Figs. 7 and 8).

DISCUSSION

The light microscopical appearances of established friction blisters reported in this paper, are similar to those reported by other workers (Naylor, 1955; Sulzberger *et al.*, 1966; Fukuyama & Cortese, 1968; Cortese *et al.*, 1969) in that they appear in the upper Malpighian layer. Earlier events, however, seem not to have been recorded previously. This work shows that changes during the pre-blistering phase can be seen provided they are searched for in numerous sections. The electron microscopic appearances suggest that they consist of keratinocyte intracellular oedema, membranous damage and irregular dilatation of the extracellular space. Cell contents then pour out into the extracellular space and the remains of the cells become necrotic. A small intra-epidermal blister (Fig. 1) is formed and this presumably enlarges (Fig. 2) when continuing friction affects neighbouring cells. With further friction the roof of the blister is rubbed off, leaving an erosion.

There is a sharp contrast between the sites of epidermal injury caused by suction and friction. Large paranuclear vacuoles within keratinocytes are an early feature of suction injury, whilst the earliest changes with friction are seen mainly in relation to keratinocyte membranes. The dermo-epidermal junction apparently resists frictional better than suction injury, and as a result blistering occurs at different levels, i.e. intra-epidermal friction blister and subepidermal suction blister.

In dermographism, a frictional force applied to the skin produces a sequence of events probably mediated by histamine release from dermal mast cells. As a result of increased permeability of the superficial dermal vessels, the upper dermis and the epidermis are flooded with increased intercellular fluid. Cauna, Macy & Cralley (1970) have studied skin ultrastructure in dermographic weals. It is tempting to suggest that the changes they noted, which were similar to those reported in this paper, were due to frictional forces, and that the paranuclear vacuoles also reported were due to the increased flow of intercellular fluids (as in suction).

The ultrastructural sites of damage caused by suction and by friction are in turn different from those in other experimental models used for the production of blistering. Low concentrations ($<0{\cdot}4\%$) of collagenase cause a clean epidermal–dermal separation in guinea-pig skin by directly damaging the basal lamina (Kahl & Pearson, 1967b), whilst higher concentrations ($2{\cdot}0\%$) for a longer period produce, in addition, damage to upper dermal collagen. With a short exposure to papain there is dermo-epidermal separation at a level either between the basal lamina and basal cell or deep to the basal lamina, but with a longer exposure there is epidermal acantholysis due to symmetrical cleavage of the desmosomes (Miller & Stoughton, 1960; Kahl & Pearson, 1967a). Cantharidin likewise produces an acantholytic blister within the epidermis, but this is apparently an enzyme-dependent reaction (Stoughton & Bagatell, 1959). Mild to moderate thermal burns, however, produce appearances similar to those we have observed following friction trauma. In both there are intracellular oedema, early loss of organelles, clumping of tonofilaments, and leakage of cytoplasm through ruptured plasma membranes into the intercellular space (Pearson, 1965). Nevertheless, caution should be exercised before inferring that friction damages cells by a local rise in temperature. Naylor (1955), by varying the speed of rubbing and by using heads of different thermal conductivity, concluded that the damage was not temperature-dependent, but more likely due to mechanical distortion of the cells. The observations reported here are quite consistent with such a view.

ACKNOWLEDGMENTS

This study was supported in part by a Grant from the Scottish Hospital Endowment Research Trust (HERT 332). It is a pleasure to acknowledge the skilful technical assistance of D.J.Fairley and to thank Professor G.J.Romanes and his staff for the use of the electron microscope in the Department of Anatomy, University of Edinburgh.

REFERENCES

CAUNA, N., MACY, I.L. & CRALLEY, A. (1970) The fine morphology of the human skin in dermographism *Journal of Allergy*, **45**, 266.

COMAISH, J.S. (1973) Glutaraldehyde lowers skin friction and enhances skin resistance to acute friction injury. *Acta dermato-venereologica*, **53**, in press.

CORTESE, T.A. JR, GRIFFIN, T.B., LAYTON, L.L. & HUTSELL, T.C. (1969) Experimental friction blisters in macaque monkeys. *Journal of Investigative Dermatology*, **53**, 172.

EPSTEIN, W.L., FUKUYAMA, K. & CORTESE, T.A. (1969) Auto-radiographic study of friction blisters. RNA, DNA and protein synthesis. *Archives of Dermatology*, **99**, 94.

FUKUYAMA, K. & CORTESE, T.A. (1968) In: *Proceedings of the 13th International Congress of Dermatology* (Ed. by W.Jadassohn and C.G.Schirren), p. 1288. Springer, Berlin.

HUNTER, J.A.A., MCVITTIE, E. & COMAISH, J.S. (1974) Light and electron microscopic studies of physical injury to the skin. I. Suction. *British Journal of Dermatology*, **90**, 481.

KAHL, F.R. & PEARSON, R.W. (1967a) Ultrastructural studies of experimental vesiculation. I. Papain. *Journal of Investigative Dermatology*, **49**, 43.

KAHL, F.R. & PEARSON, R.W. (1967b) Ultrastructural studies of experimental vesiculation. II. Collagenase. *Journal of Investigative Dermatology*, **49**, 616.

MILLER, R.F. & STOUGHTON, R.B. (1960) Enzymatic vesication *in vivo*. I. Effects of papain in human skin. *Journal of Investigative Dermatology*, **35**, 141.

NAYLOR, P.F.D. (1955) Experimental friction blisters. *British Journal of Dermatology*, **67**, 327.

PEARSON, R.W. (1965) Response of human epidermis to graded thermal stress: a morphologic comparison of burns, cold-induced blisters, and pemphigus vulgaris. *Archives of Environmental Health*, **11**, 498.

STOUGHTON, R.B. & BAGATELL, F. (1959) The nature of Cantharidin acantholysis. *Journal of Investigative Dermatology*, **33**, 287.

SULXBERGER, M.B., CORTESE, T.A., FISHMAN, L. & WILEY, H.S. (1966) Studies on blisters produced by friction. I. Results of linear rubbing and twisting techniques. *Journal of Investigative Dermatology*, **47**, 456.

British Journal of Dermatology (1976) **94**, 119.

Clinical and Laboratory Investigations

Langerhans cells in the epidermis of athymic mice

J.A.A.HUNTER, D.J.FAIRLEY, G.C.PRIESTLEY AND H.A.CUBIE

Department of Dermatology, University of Edinburgh, The Royal Infirmary, Edinburgh, Scotland

Accepted for publication 5 September 1975

SUMMARY

Langerhans cells were identified with the electron microscope in the epidermis of six athymic *nu/nu* mice. Their origin in the thymus is ruled out and their functional dependence on either the thymus or T lymphocytes seems improbable.

The origin and function of the epidermal Langerhans cell are still unknown. Apparently identical cells occur in extra-epidermal situations including lymph nodes (Jimbow, Sato & Kukita, 1969; Kondo, 1969) and the thymus (Haelst, 1969), and since Wolff's recent comprehensive review of the cell (Wolff, 1972) attention has been drawn to the possibility of an immunological role for the cells in contact allergy (Silberberg, Baer & Rosenthal, 1974). It therefore seemed worth while to find out whether Langerhans cells were present in the epidermis of athymic mice, in which T lymphocytes are virtually absent and delayed hypersensitivity reactions, including contact allergy, are abolished (Pantelouris, 1973).

MATERIALS AND METHODS

The mice were three black and three albino male nude homozygotes (*nu/nu*), 6–7 weeks old, raised under specific pathogen-free conditions at the Moredun Institute for Animal Diseases, Edinburgh. Mice from this stock have the typical nude phenotype (Flanagan, 1966) and accept grafts of human skin (Cubie, 1972). Post mortem examination of each mouse confirmed the absence of a thymus.

The mice were killed by cervical dislocation and pieces of mid-dorsal skin were immediately immersed in half-strength Karnovsky's fixative (Karnovsky, 1965), cut into small blocks and fixed for 5 h at room temperature. After post-fixation in 1% aqueous osmium tetroxide, specimens were stained *en bloc* with 2% aqueous uranyl acetate, and embedded in araldite. Thick sections stained with toluidine blue were examined and thin sections of selected areas of epidermis containing dendritic cells were cut with an LKB III ultra microtome. Sections on grids were stained with uranyl acetate and lead citrate and examined using an A.E.I. Corinth at 50 kv.

RESULTS

Epidermal Langerhans cells were found without difficulty in each of the six mice (Fig. 1). In one mouse a Langerhans cell was seen in the dermis. The granules in the Langerhans cells appeared normal;

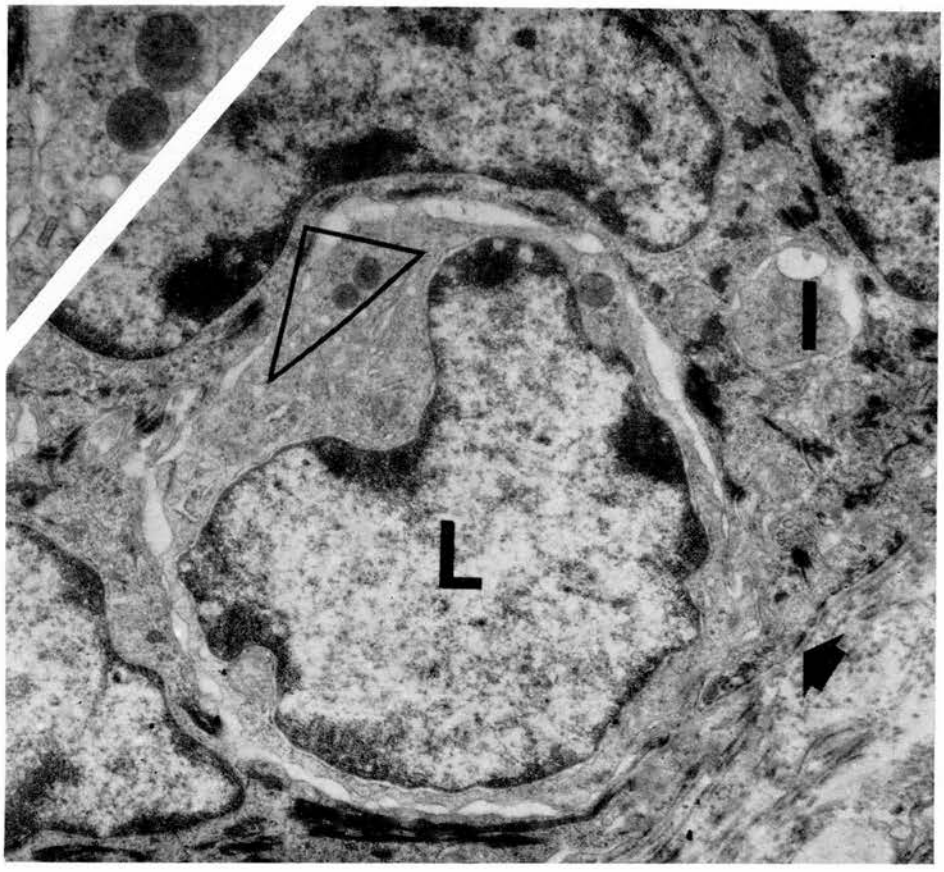


FIGURE 1. Langerhans cell (L) and its dendritic process (l) between keratinocytes of the basal cell layer. Arrow points to basal lamina (× 13,500). Inset (area outlined in triangle) shows a granule and two lysosomes (× 35,000).

both rod and racket shaped profiles were noted (Fig. 2) and one was seen in contact with the cell wall. Other intracellular organelles including mitochondria, ribosomes, Golgi and endoplasmic reticulum were normal, though lysosomes were particularly numerous in some cells. Centrioles were also seen. The appearance of the Langerhans cells therefore indicated an active cell population. The ultrastructure of the rest of the epidermis, although not studied in detail, seemed unremarkable.

COMMENT

The origin and function of the epidermal Langerhans cell are still unknown (Wolff, 1972). Wolff favoured a mesodermal origin. Silberberg *et al.* (1974) suggested an immunological function in contact allergy reactions, where a close relationship with mononuclear cells (in this case, T lymphocytes) is envisaged and the Langerhans cell might process antigen like the macrophage.

Cells containing Langerhans granules have been noted in the thymus (Haelst, 1969), an organ which produces and influences the function of T lymphocytes. If there is a close relationship between Langerhans cells and T lymphocytes, as suggested by Silberberg, Langerhans cells could also originate

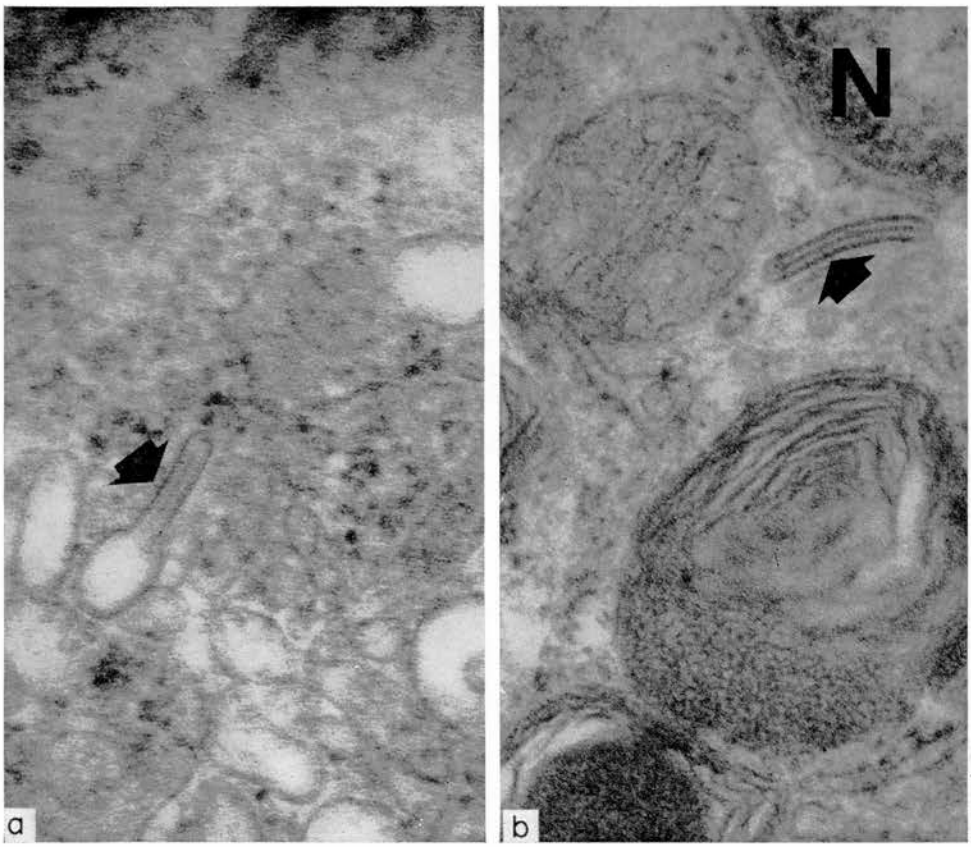


FIGURE 2. (a) Racket shaped granule (arrow) (×92,000). (b) Rod shaped granule (arrow) below which are seen two lysosomes. N, nucleus (×81,500).

in the thymus, or be dependent on it. Langerhans cells appear in human embryonic skin at 14 weeks (Breathnach & Wyllie, 1965) while the thymus develops earlier at about 9 weeks (Hamilton, Boyd & Mossman, 1972), so a thymic origin for Langerhans cells is possible.

In nude mice the thymus never reaches the lymphoid state and remains as a cystic rudiment. As a consequence immunological competence is not achieved (Pantelouris, 1973). Circulating lymphocytes are reduced 5–6 fold and at least 97% of those remaining are B cells (Sprent & Miller, 1972). In fact many nude animals appear to have no T cells at all when examined by the sheep red blood cell rosette test. Delayed hypersensitivity reactions, including contact dermatitis, are abolished. Grafts of foreign tissue are accepted.

We believe that the finding of Langerhans cells in athymic mice shows that the epidermal Langerhans cell: (1) does not originate in the thymus, and (2) on structural evidence alone, is not dependent on the thymus or T lymphocytes.

This does not preclude a close functional relationship between T cells and Langerhans cells, as suggested by Silberberg *et al.* (1974).

It will be important to determine whether the Langerhans cell population in athymic mice differs in size from that in normal mice. Histochemical studies with that aim are in progress.

ACKNOWLEDGMENT

This work was carried out with support from the Scottish Hospital Endowments Research Trust.

REFERENCES

BREATHNACH, A.S. & WYLLIE, L.M.A. (1965) Electron microscopy of melanocytes and Langerhans cells in human fetal epidermis at fourteen weeks. *Journal of Investigative Dermatology*, **44**, 51.

CUBIE, H.A. (1972) Laboratory investigations in the study of human papilloma virus. *M.Sc. thesis*, University of Edinburgh.

FLANAGAN, S.P. (1966) 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genetic Research*, **8**, 295.

HAELST, U.J.C. VAN (1969) Light and electron microscopic study of the normal and pathological thymus of the rat. III. A mesenchymal histiocytic type of cell. *Zeitschrift für Zellforschung und mikroskopische Anatomie*, **99**, 198.

HAMILTON, W.J., BOYD, J.D. & MOSSMAN, H.W. (1972) *Human Embryology*, 4th edn, p. 319. Heffer, Cambridge.

JIMBOW, K., SATO, S. & KUKITA, A. (1969) Cells containing Langerhans granules in human lymph nodes of dermatopathic lymphadenopathy. *Journal of Investigative Dermatology*, **53**, 295.

KARNOVSKY, M.J. (1965) A formaldehyde–glutaraldehyde fixative of high osmolarity for use in electron microscopy. *Journal of Cell Biology*, **27**, 137A.

KONDO, Y. (1969) Macrophages containing Langerhans cell granules in normal lymph nodes of the rabbit. *Zeitschrift für Zellforschung und mikroskopische Anatomie*, **98**, 506.

PANTELOURIS, E.M. (1973) Athymic development in the mouse. *Differentiation*, **1**, 437.

SILBERBERG, I., BAER, R.L. & ROSENTHAL, S.A. (1974) The role of Langerhans cells in contact allergy. *Acta dermato-venereologica*, **54**, 321.

SPRENT, J.R. & MILLER, J.F.A.P. (1972) Thoracic duct lymphocytes from nude mice: migratory properties and life span. *European Journal of Immunology*, **2**, 384.

WOLFF, K. (1972) The Langerhans Cell. In: *Current Problems in Dermatology*, Vol. 4 (ed. by J.W.H. Mali), p. 79. Karger, Basel.